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**EXPANSÃO, CRIOPRESERVAÇÃO E SINCRONIZAÇÃO EM G₀/G₁ DE
FIBROBLASTOS PARA APLICAÇÕES BIOTECNOLÓGICAS VISANDO A
CONSERVAÇÃO DO GATO-DO-MATO-PEQUENO (*Leopardus tigrinus* SCHREBER,
1775)**

Mestrando: João Vitor da Silva Viana

Orientadora: Prof. Dra. Alexsandra Fernandes Pereira

MOSSORÓ–UFERSA

2025

JOÃO VITOR DA SILVA VIANA

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Dissertação apresentada à Universidade Federal Rural do Semi-Árido (UFERSA), como exigência final para obtenção do título de Mestre no Curso de Pós-Graduação em Ciência Animal.

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal.

Orientadora: Profª. Dra. Alexsandra Fernandes Pereira

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JOÃO VITOR DA SILVA VIANA

**EXPANSÃO, CRIOPRESERVAÇÃO E SINCRONIZAÇÃO EM G₀/G₁ DE
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BANCA EXAMINADORA

Profa. Dra. Alexsandra Fernandes Pereira (UFERSA)
Presidente

Profa. Dra. Fabiana Fernandes Bressan (USP)
Membro examinador

Prof. Dr. Carlos Eduardo Bezerra de Moura (UFERSA)
Membro examinador

DADOS CURRICULARES DO AUTOR

JOÃO VITOR DA SILVA VIANA – Nascido em Fortaleza, CE, dia 19 de agosto de 2001, filho de Maria Meryanne da Silva Cartaxo e Francisco Andrade Viana. Graduando-se em Biotecnologia pela Universidade Federal Rural do Semi-Árido (UFERSA, 2019–2022). Estagiou no Laboratório de Biotecnologia Animal (LBA/UFERSA), participando do Programa Institucional Voluntário de Iniciação Científica (UFERSA, 2019–2020) e do Programa Institucional de Bolsas de Iniciação Científica do Conselho Nacional de Desenvolvimento Científico e Tecnológico (UFERSA/CNPq, 2020–2022), bem como auxiliou em diferentes experimentos relacionados à criopreservação de células e tecidos somáticos, e cultivo *in vitro*. Em dezembro de 2022, foi aprovado no processo seletivo para o mestrado acadêmico em Ciência Animal (PPGCA/UFERSA), iniciando em março de 2023 as atividades no Laboratório de Biotecnologia Animal (LBA/UFERSA) com bolsa de auxílio financeiro pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

*Para minha mãe Maria Meryanne da Silva
Cartaxo, meu padrasto Francisco Soares
Cartaxo, meus familiares e amigos por todo
apoio e confiança. Dedico.*

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*“O que é verdadeiramente imoral é ter desistido
de si mesma.”*

Clarice Lispector

Viana, João Vitor da Silva. EXPANSÃO, CRIOPRESERVAÇÃO E SINCRONIZAÇÃO EM G₀/G₁ DE FIBROBLASTOS PARA APLICAÇÕES BIOTECNOLÓGICAS VISANDO A CONSERVAÇÃO DO GATO-DO-MATO-PEQUENO (*Leopardus tigrinus* SCHREBER, 1775). 2025. (Mestrado em Ciência Animal: Morfofisiologia e Biotecnologia Animal) Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, 2025.

RESUMO: O gato-do-mato-pequeno é um mesopredador que auxilia no equilíbrio de seu habitat por meio do controle de suas presas. Contudo, ele tem sofrido constantes ameaçadas, sendo considerado uma espécie vulnerável à extinção. Neste contexto, bancos de células somáticas associados às ferramentas de reprogramação nuclear tornam-se essenciais para a conservação da espécie. Portanto, o objetivo foi comparar o efeito do número de passagens, crioprotetores e métodos de sincronização em G₀/G₁ de fibroblastos de gato-do-mato-pequeno. Para tanto, células somáticas derivadas da pele de quatro animais provenientes de zoológicos, foram identificadas como fibroblastos por ensaios de imunocitoquímica e com cariótipo normal por cariotipagem. Em seguida, fibroblastos foram distribuídos em três experimentos. No **primeiro experimento**, fibroblastos foram cultivados por diferentes passagens (terceira, sexta e décima primeira) e submetidos à criopreservação. No **segundo experimento**, células foram criopreservadas por congelamento lento em diferentes concentrações de dimetilsulfóxido (2,5 e 10% de DMSO), na ausência e presença de 0,2 M de sacarose ou 100 mM de L-prolina, e avaliadas antes e após a criopreservação. Em ambos os experimentos, células foram avaliadas quanto à morfologia, viabilidade, atividade metabólica, atividade proliferativa, níveis das espécies reativas de oxigênio (EROs), potencial de membrana mitocondrial ($\Delta\Psi$ m), e níveis apoptóticos. Exclusivamente no primeiro experimento, células foram avaliadas em sistema sem antibiótico, e análise ultraestrutural. No **terceiro experimento**, fibroblastos foram submetidos à sincronização em G₀/G₁, onde foram avaliados após diferentes condições [incubação com roscovitina a 7,5 e 15 μ M (24 e 48 h), inibição por contato (24–72 h) e privação de soro fetal bovino (24–96 h)]. Neste experimento, células foram avaliadas quanto à morfologia, viabilidade, níveis de apoptose e capacidade de sincronização em G₀/G₁. No primeiro experimento, foi observada uma redução na atividade metabólica e $\Delta\Psi$ m na sétima e décima-primeira passagem, bem como a décima primeira passagem resultou na redução da proliferação celular e modificações morfológicas. Demais parâmetros não foram alterados. No segundo experimento, 10% de DMSO apresentou os melhores resultados promovendo manutenção da viabilidade imediatamente após a descongelamento e níveis de EROs. No terceiro experimento, a privação de soro fetal bovino foi o único método que promoveu aumento na taxa de células em G₀/G₁ de 86,0% $\pm$ 0,9 para 91,4% $\pm$ 1,7; contudo, ele reduziu o número de células viáveis de 99,8% $\pm$ 0,3 para 93,3% $\pm$ 0,6. Os demais grupos [roscovitina e inibição por contato] não aumentaram as taxas de células em G₀/G₁. Em conclusão, células de gato-do-mato-pequeno apresentaram danos em sua proliferação a partir da sétima passagem. Ainda, tais células demonstraram que podem ser criopreservadas com 10% de DMSO, apresentando alta viabilidade após a descongelamento. Finalmente, a privação com soro demonstrou ser o método capaz de sincronizar células em G₀/G₁. Adicionalmente, o estabelecimento dessas etapas possibilitará os próximos passos para aplicação de células de gato-do-mato-pequeno visando sua conservação.

Palavras-chave: pequenos felídeos, criobancos, células somáticas, privação de soro fetal bovino

Viana, João Vitor da Silva. EXPANSION, CRYOPRESERVATION, AND G₀/G₁ SYNCHRONIZATION OF FIBROBLASTS FOR BIOTECHNOLOGICAL APPLICATIONS TARGETING THE CONSERVATION OF THE NORTHERN TIGER CAT (*Leopardus tigrinus*, Schreber, 1775). 2025. (Master's in Animal Science: Animal Morphophysiology Animal Biotechnology) Federal Rural University of the Semi-Arid (UFERSA), Mossoró, RN, 2025.

ABSTRACT: The northern tiger cat is a mesopredator that contributes to the equilibrium of its habitat by the prey population control. However, it has been subjected to constant threats and is considered vulnerable to extinction. In this context, somatic cell banks combined with nuclear reprogramming tools are essential for the conservation of the species. Therefore, the aim was to compare the effect of passages number, cryoprotectants, and G₀/G₁ synchronization methods in fibroblasts from the northern tiger cat. Then, somatic cells derived from the skin of four animals from zoos were identified as fibroblasts through immunocytochemistry assays and regular karyotype by karyotyping. Subsequently, fibroblasts were distributed into three experiments. In **the first experiment**, fibroblasts were cultured at different passages (third, sixth, and eleventh) and subjected to cryopreservation. In the **second experiment**, cells were cryopreserved using slow freezing with different concentrations of dimethyl sulfoxide (2.5% and 10% DMSO) in the absence and presence of 0.2 M sucrose or 100 mM L-proline and evaluated before and after cryopreservation. In both experiments, cells were assessed for morphology, viability, metabolic activity, proliferative activity, levels of reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi_m$), and apoptotic levels. Exclusively in the first experiment, cells were evaluated in an antibiotic-free system and via ultrastructural analysis. In the **third experiment**, fibroblasts were subjected to G₀/G₁ synchronization under different conditions [incubation with roscovitine at 7.5 and 15 μ M (24 and 48 h), contact inhibition (24–72 h), and fetal bovine serum starvation (24–96 h)]. In this experiment, cells were evaluated for morphology, viability, apoptosis levels, and G₀/G₁ synchronization efficiency. In the first experiment, a reduction in metabolic activity and $\Delta\Psi_m$ was observed at the seventh and eleventh passages, with the eleventh passage showing decrease in cell proliferation and morphological alterations. Other parameters were not changed. In the second experiment, 10% DMSO showed the best results, maintaining viability immediately after thawing and ROS levels. In the third experiment, fetal bovine serum starvation was the only group that increased the proportion of cells in G₀/G₁ from 86.0% $\pm$ 0.9 to 91.4% $\pm$ 1.7; although, it reduced the number of viable cells from 99.8% $\pm$ 0.3 to 93.3% $\pm$ 0.6. The other groups [roscovitine and contact inhibition] did not increase the cell rates in G₀/G₁. In conclusion, northern tiger cat cells showed proliferative damages at the seventh passage. Furthermore, these cells demonstrated that they could be cryopreserved with 10% DMSO, maintaining high viability after thawing. Finally, serum starvation proved to be the method capable of synchronizing cells in G₀/G₁. Additionally, the establishment of these steps will enable the next steps for the application of small wildcat cells aiming at their conservation.

Keywords: small felids, cryobanks, somatic cells, fetal bovine serum starvation

LISTA DE FIGURAS

CAPÍTULO 1-CONSIDERAÇÕES GERAIS 24

Figura 1. Exemplar de gato-do-mato-pequeno em confinamento no ECOPOINT Parque Ambiental e Zoológico. Fonte: Figura do autor. 27

CAPÍTULO 2- ESTABLISHMENT AND CHARACTERIZATION OF FIBROBLAST LINES FROM THE NORTHERN TIGER CAT (*Leopardus tigrinus*, SCHREBER, 1775) DURING EXTENDED PASSAGE AND CRYOPRESERVATION 52

Figure 1. Cell type characterization using immunophenotyping and karyotyping assays in northern tiger cat. (A) Fibroblasts of one animal (A2, male) stained with vimentin (green) identifying the cytoplasmatic projection. (B) The same fibroblasts of animal stained with Hoechst 33342 (blue) identifying the nucleus. Scale bar 50 μ m, 40 $\times$. (C) Overlapping stained nucleus and vimentin. (D, E) Karyotype of one animal (A2, male) in the third passage, n = 36. Scale bar 100 μ m, 100 $\times$ 63

Figure 2. Evaluation of morphology of fibroblasts derived from northern tiger cat after extended cell culture. Fibroblasts during (A) primary cell culture, (B) third, (C) seventh, (D) eleventh passage. Scale bar 50 μ m, 20 $\times$ 64

Figure 3. Viability and proliferation of cells derived from northern tiger cat cell after extended cell culture (third, seventh, and eleventh passages). (A) Cell viability by trypan blue assay. (B) Metabolic activity determined by the MTT assay. Apoptosis levels by identification with acridine orange and ethidium bromide after (C) third, viable cells (Circle); Necrotic cells (Arrow), (C') seventh, viable cells (Circle); Initial apoptotic (Square), and (C'') eleventh passage, viable cells (Circle); Initial apoptotic (Square); Later apoptotic (Triangle); Necrotic cells (Arrow). (D)

Apoptotic levels quantification. (E) Population double time (PDT) after seven days of cell culture. (F) Growth curve after seven days of cell culture. Scale bars 50 μm , 40 $\times$ (C, C' and C'').
^{a,b}: Values with different superscript represent significant differences ($P < 0.05$). 65

Figure 4. Oxidative stress and potential mitochondrial membrane after third, seventh, and eleventh passages in cells derived from northern tiger cat. Marked cells with H₂DCFDA during (A) third, (B) seventh and (C) eleventh passage. (D) ROS level quantification in arbitrary fluorescence units. Image of potential mitochondrial membrane marked with MitoTracker Red during (E) third, (F) seventh and (G) eleventh passages. (H) Potential mitochondrial membrane using arbitrary fluorescence units measurement; Scale bars 50 μm , 40 $\times$; ^{a,b}: Values with different superscript represent significant differences ($P < 0.05$). 66

Figure 5. Morphology and ultrastructure of fibroblasts after cryopreservation derived from northern tiger cat. Morphology of (A) non-cryopreserved and (B) cryopreserved fibroblasts under an inverted microscope. Scale bar: 50 μm , 20 $\times$. (A') Ultrastructure of non-cryopreserved and (B') cryopreserved cells with a fusiform shape and cell projections. Scale bar: 5 μm 67

Figure 6. Evaluation of cell parameters of northern tiger cat cells after cryopreservation. (A) Viability measured with trypan blue. (B) Metabolic activity measurement with MTT assay. Cells of third passage marked with acridine orange and ethidium bromide (C) Viable cells, (D) initial apoptotic, (E) late apoptotic (F) necrotic cells. (G) Quantification of apoptosis levels. (H) Growth curve. (I) Population double time; (C, D, E, and F) Scale bars 50 μm , 100 $\times$. $P > 0.05$. .. 68

Figure 7. Evaluation of ROS levels by H₂DCFDA and mitochondrial membrane potential with MitoTracker Red after northern tiger cat cells cryopreservation. (A) Non-cryopreserved and (A') cryopreserved cells marked with H₂DCFDA. (B) Measurement of arbitrary fluorescence units for ROS levels quantification; (C) Non-cryopreserved and (C') cryopreserved cells marked with MitoTracker Red. (D) mitochondrial membrane potential evaluation by arbitrary fluorescence

unity quantification. Scale bars 50 μm , 40 $\times$. ^{a,b}: Values with different superscript represent significant differences ($P < 0.05$). 69

CAPÍTULO 3- NO SYNERGISTIC EFFECT OF EXTRACELLULAR CRYOPROTECTANTS WITH DIMETHYL SULFOXIDE IN THE CONSERVATION OF NORTHERN TIGER CAT FIBROBLASTS 79

Figure 1. Morphology evaluation of northern tiger cat fibroblasts subjected to cryopreservation in different solutions. (A) Non-cryopreserved cells and cells cryopreserved using (B) 2.5% Me₂SO; (C) 10% Me₂SO; (D) 2.5% Me₂SO with sucrose; (E) 2.5% Me₂SO with L-proline; (F) 10% Me₂SO with sucrose; (G) 10% Me₂SO with L-proline; Scale bars: 50 μm . Magnification: 20 $\times$ 91

Figure 2. Membrane integrity and proliferative and metabolic activities of northern tiger cat fibroblasts after cryopreservation using different cryoprotectant solutions. (A) Cell membrane integrity; (B) Population double time (PDT); (C) Growth curve after 7 days of cell culture; (D) Metabolic activity. ^{a,b}: $P < 0.05$ 91

Figure 3. Measurement of apoptotic levels of northern tiger cat fibroblasts after cryopreservation using different cryoprotectant solutions. Representative image of non-cryopreserved cells (A) and cells cryopreserved with 2.5% Me₂SO (B); 10% Me₂SO (C); 2.5% Me₂SO with sucrose (D); 10% Me₂SO with sucrose (E); 2.5% Me₂SO with L-proline (F); 10% Me₂SO with L-proline (G); Circle: Viable cell; Square: Initial apoptosis; Triangle: Late apoptosis; Arrow: Necrosis. (E) Quantification of apoptotic levels; Scale bars: 50 μm . Magnification: 40 $\times$ 92

Figure 4. Oxidative stress measured by the ROS levels and mitochondrial membrane potential ($\Delta\Psi\text{m}$) of northern tiger cat fibroblasts after cryopreservation using different cryoprotectant solutions. (A) Non-cryopreserved cells marked with MitoTracker Red; (B) Mitochondrial

membrane potential measurement; (C) Non-cryopreserved cells marked with H₂DCFDA; (D) Quantification of ROS levels. Scale bars: 50 µm. Magnification: 40×. ^{a,b,c} P < 0.05..... 93

CAPÍTULO 4- THE ROLE OF SOMATIC CELL SYNCHRONIZATION IN NUCLEAR TRANSFER AND INDUCED PLURIPOTENT STEM CELLS FOR WILD FELIDS..... 102

Figure 1. Representative scheme of cells cycling and cells subjected to synchronization in G₀/G₁.....108

Figure 2. Studies with somatic tissue and cells in wild felids. 117

CAPÍTULO 5- EXPLORING THE EFFECTS OF ROSCOVITINE, SERUM STARVATION, AND CONTACT INHIBITION ON G₀/G₁ ARREST IN NORTHERN TIGER CAT DERMAL FIBROBLASTS 130

Figure 1. Experimental design of northern tiger cat fibroblasts treatments and analysis..... 137

Figure 2. Representative histograms after analysis by Guava Easycyte flow cytometer of northern tiger cat fibroblasts subjected to RSV, CI and SS. 138

Figure 3. Evaluation of northern tiger cat cells before treatment of cell arrest G₀/G₁. **A)** Morphology of fibroblasts with 50–60% of confluence; **B)** Apoptotic levels evaluation with viable cells (green); **C)** Growth curve of each animal under 7 days (168 h) of cell culture. Scale bar: 50 µm; Magnification: 20×; 140

Figure 4. Morphology evaluation of northern tiger cat after RSV treatment; Magnification 20×:

A) RSV 7.5 μ M 24 h; B) RSV 15 μ M 24 h; C) RSV 7.5 μ M 48 h; D) RSV 15 μ M 48 h; Magnification 40 $\times$: A') RSV 7.5 μ M 24 h; B') RSV 15 μ M 24 h; C') RSV 7.5 μ M 48 h; D') RSV 15 μ M 48 h; Scale bar: 50 μ m; 141

Figure 5. Evaluation of viability and apoptotic levels of northern tiger cat after RSV treatment; A) Viability measurement by trypan blue assay; Evaluation of apoptotic levels with acridine orange and ethidium bromate: B) RSV 7.5 μ M 24 h; C) RSV 15 μ M 24 h; D) RSV 7.5 μ M 48 h; E) RSV 15 μ M 48 h; F) Percentage of apoptotic levels Scale bar: 50 μ m; ^{a,b}: values with different superscript represent significant differences (P <0.05). 142

Figure 6. Morphology evaluation of northern tiger cat cells after SS treatment; Magnification 20 $\times$: A) SS 24 h; B) SS 48 h; C) SS 72 h; D) SS 96 h; Magnification 40 $\times$: A') SS 24h; B') SS 48 h; C') SS 72 h; D') SS 96 h Scale bar: 50 μ m;..... 144

Figure 7. Evaluation of viability and apoptotic levels of northern tiger cat after SS treatment; A) Viability measurement by trypan blue assay after SS; Evaluation of apoptotic levels with acridine orange and ethidium bromate: B) SS 24 h; C) SS 48 h; D) SS 72 h; E) SS 96 h; F) Percentage of viable, initial apoptotic, late apoptotic and necrotic cells. Scale bar: 50 μ m; ^{a,b}: values with different superscript represent significant differences (P <0.05). 144

Figure 8. Morphology evaluation of northern tiger cat after CI treatment; Magnification 20 $\times$: A) CI 24h; B) CI 48h; C) CI 72h; Magnification 40 $\times$: A') CI 24h; B') CI 48h; C') CI 72h; Scale bar: 50 μ m;.....146

Figure 9. Evaluation of viability and apoptotic levels of northern tiger cat after CI treatment; A) Viability measurement by trypan blue assay; Evaluation of apoptotic levels with acridine orange and ethidium bromate: B) CI 24h; C) CI 48h; E) CI 72h; F) Percentage of viable, initial apoptotic, late apoptotic and necrotic cells. Scale bar: 50 μ m; absence of superscript represents no

significant differences ($P > 0.05$).	147
---	-----

LISTA DE TABELAS

CAPÍTULO 2- ESTABLISHMENT AND CHARACTERIZATION OF FIBROBLAST LINES FROM THE NORTHERN TIGER CAT (*Leopardus tigrinus*, SCHREBER, 1775) DURING EXTENDED PASSAGE AND CRYOPRESERVATION 52

Table 1. Description of primary cell culture of somatic tissues and cells derived from northern tiger cat. 62

CAPÍTULO 4- THE ROLE OF SOMATIC CELL SYNCHRONIZATION IN NUCLEAR TRANSFER AND INDUCED PLURIPOTENT STEM CELLS FOR WILD FELIDS 102

Table 1. Difference between the culture conditions and cell proliferation of somatic cells derived from wild felids. 109

Table 2. Studies using chemical agents in wild felid cells for G₀/G₁ cycle synchronization. 114

Table 3. Effect of SS and CI on G₀/G₁ cells of wild felids. 118

CAPÍTULO 5- EXPLORING THE EFFECTS OF ROSCOVITINE, SERUM STARVATION, AND CONTACT INHIBITION ON G₀/G₁ ARREST IN NORTHERN TIGER CAT DERMAL FIBROBLASTS 130

Table 1. Effect of concentration and time of exposure of RSV on the percentage of northern tiger cat fibroblasts in the G₀/G₁, S, and G₂/M phases of the cycle. 142

Table 2. Effect of SS on the percentage of northern tiger cat fibroblasts in the G₀/G₁, S, and G₂/M phases of the cycle. 145

Table 3. Effect of CI on the percentage of northern tiger cat fibroblasts in the G ₀ /G ₁ , S, and G ₂ /M phases of the cycle.	147
---	------------

LISTA DE QUADRO

CAPÍTULO 1-CONSIDERAÇÕES GERAIS	24
Quadro 1. Conservação usando congelação lenta de células somáticas em felídeos silvestres. ..	31
Quadro 2. Condições de cultivo <i>in vitro</i> de células somáticas de felídeos silvestres.	33

LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de variância
ATP	Adenosina trifosfato
CEUA	Comissão Ética no Uso de Animais
cm	Centímetro
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO ₂	Dióxido de Carbono
DMEM	Meio Essencial Mínimo Modificado por Dulbecco
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
E	Epiderme
EP	Erro Padrão
h	Hora
HE	Hematoxilina-eosina
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
iPS	Célula-tronco de pluripotência induzida
IUCN	International Union for Conservation of Nature
LBA	Laboratório de Biotecnologia Animal
M	Molar
mg/kg	Miligrama por quilo
min	Minuto
mL	Militro
mL/kg	Mililitro por quilo

Mm	Milímetro
mm ²	Milímetro quadrado
mm ³	Milímetro cúbico
MTT	Brometo de 3-4,5-dimetil-tiazol-2-il-2,5-difeniltetrazólio
nm	Nanômetro
N	Número
nº	Número
°C	Graus Celsius
PBS	Solução tampão fosfato
PDT	Tempo de duplicação
pH	Potencial hidrogeniônico
RN	Rio Grande do Norte
RNA	Ácido ribonucleico
SAC	Sacarose
SFB	Soro Fetal Bovino
TNCS	Transferência Nuclear de Célula Somática
UFERSA	Universidade Federal Rural do Semi-Árido
v	Volume
µL	Microlitros
µm	Micrômetro
µm ²	Micrômetro quadrado

SUMÁRIO

CAPÍTULO 1-CONSIDERAÇÕES GERAIS	24
1. INTRODUÇÃO	24
2. FUNDAMENTAÇÃO TEÓRICA.....	26
2.1. RELEVÂNCIA ECOLÓGICA E DESCRIÇÃO POPULACIONAL DO GATO-DO-MATO-PEQUENO.....	26
2.2. ESTRATÉGIAS DE CONSERVAÇÃO PARA O GATO-DO-MATO-PEQUENO.....	27
2.3. BANCOS SOMÁTICOS PARA A CONSERVAÇÃO DE FELÍDEOS SILVESTRES	29
2.3.1. Identificação do tipo celular e estabelecimento das condições de cultivo <i>in vitro</i>.....	30
2.3.2. Determinação das condições de criopreservação de células somáticas	32
2.3.3. Avaliação funcional das células somáticas usando sistemas de sincronização em G₀/G₁.....	35
3. JUSTIFICATIVA	37
4. HIPÓTESES	38
5. OBJETIVOS	39
5.1. OBJETIVO GERAL	39
5.2. OBJETIVOS ESPECÍFICOS.....	39
6. REFERÊNCIAS	40
CAPÍTULO 2- ESTABLISHMENT AND CHARACTERIZATION OF FIBROBLAST LINES FROM THE NORTHERN TIGER CAT (<i>Leopardus tigrinus</i>, SCHREBER, 1775) DURING EXTENDED PASSAGE AND CRYOPRESERVATION	52
CAPÍTULO 3- NO SYNERGISTIC EFFECT OF EXTRACELLULAR CRYOPROTECTANTS WITH DIMETHYL SULFOXIDE IN THE CONSERVATION OF NORTHERN TIGER CAT FIBROBLASTS	79
CAPÍTULO 4- THE ROLE OF SOMATIC CELL SYNCHRONIZATION IN NUCLEAR TRANSFER AND INDUCED PLURIPOTENT STEM CELLS FOR WILD FELIDS.....	102
CAPÍTULO 5- EXPLORING THE EFFECTS OF ROSCOVITINE, SERUM STARVATION, AND CONTACT INHIBITION ON G₀/G₁ ARREST IN NORTHERN TIGER CAT DERMAL FIBROBLASTS	130
CAPÍTULO 6- CONSIDERAÇÕES FINAIS	156
ANEXOS	158
APÊNDICES.....	181

CAPÍTULO 1-CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

O Brasil apresenta grande diversidade de mamíferos, totalizando mais de 700 espécies (GALETTI et al., 2022). Destas espécies, aproximadamente, 22% encontram-se na Caatinga, onde estão inseridas as espécies pertencentes ao gênero *Leopardus*, como *L. pardalis* (SANTOS et al., 2019) *L. tigrinus*, *L. wiedii*, *L. colocola* (RAMIREZ et al., 2022). Esses animais possuem uma grande importância ecológica, sendo descritas como mesopredadores, auxiliando no equilíbrio de seus ecossistemas (KASPER et al., 2016).

Entre estas espécies, encontra-se o *L. tigrinus*, ou gato-do-mato-pequeno, considerado, segundo a União Internacional para Conservação da Natureza (IUCN), como vulnerável à extinção (PAYAN; OLIVEIRA, 2016). Entre as razões que justificam tal cenário estão os atropelamentos e a fragmentação dos habitats (SILVA et al., 2022). Neste contexto, estratégias de conservação *in situ* têm sido desenvolvidas por meio do monitoramento usando câmeras (OLIVEIRA-SANTOS et al., 2012) atrelado ao Plano Nacional para conservação de pequenos felídeos (SOUZA; BERNARD, 2020).

Além das estratégias *in situ*, abordagens *ex situ* têm sido aprimoradas, garantindo condições adequadas para a manutenção de felídeos silvestres em zoológicos e cativeiros (MOREIRA et al., 2007). Além disso, metodologias *in vitro* já foram desenvolvidas para a espécie quanto à recuperação de oócitos (PAZ et al., 2009), tecido ovariano (WIEDEMANN et al., 2013) e espermatozoides (ANGRIMANI et al., 2017). Adicionalmente, uma alternativa a estas ferramentas seria o emprego de células somáticas, a qual possui relevância quando aplicada na clonagem por transferência nuclear de célula somática (TNCS, MORO et al., 2015) e indução de células à pluripotência (IPSC, VERMA et al., 2012). Para ambas as aplicações, o estabelecimento das condições de cultivo e criopreservação é essencial para garantir a viabilidade e funcionalidade das células para esses fins.

Quanto ao estabelecimento das condições de cultivo *in vitro* em felídeos, variações na morfologia e atividade proliferativa tem sido observada ao longo do aumento do número de passagens, sendo este uma peça-chave para o uso das células. Neste sentido, espécies como o gato-pescador (*Prionailurus viverrinus*) apresentaram senescência em passagens iniciais

(terceira–quinta passagem), o que motivou o desenvolvimento de estratégias quanto à imortalização celular e suplementações do meio de cultivo (SUKPARANGSI et al., 2022). Por sua vez, células de onça-parda (*Puma concolor*) apresentaram sinais iniciais de danos após a décima passagem, sendo informações úteis para os estudos de reprogramação celular (LIRA et al., 2022). Desse modo, torna-se necessário entender os efeitos do número de passagens durante o cultivo *in vitro* na obtenção de linhagens celulares em gato-do-mato-pequeno.

Quanto às condições de criopreservação, Arantes et al. (2021) usando células somáticas de gato-do-mato-pequeno demonstraram taxas de viabilidade acima de 80% após criopreservação, usando 2,5% e 10% de dimetilsulfóxido (DMSO) suplementado com 10% de soro fetal bovino (SFB). Contudo, crioprotetores extracelulares têm sido correlacionados com redução na citotoxicidade da solução de criopreservação e maior viabilidade após criopreservação (JESUS et al., 2022). Dentre estes, pode-se citar a sacarose e L-prolina, os quais em estudos em outras espécies é relatada sua eficiência na manutenção dos parâmetros celulares além da viabilidade, tais como atividade metabólica, proliferativa e níveis de espécies reativas de oxigênio (LÉON-QUINTO et al., 2014; JESUS et al., 2022).

Além das etapas citadas, a sincronização do ciclo em G₀/G₁ é de fundamental importância para o uso em clonagem e iPSC, uma vez que representa uma etapa anterior à reprogramação nuclear (IQBAL et al., 2021). Em felídeos silvestres, resultados têm variado a depender da espécie de interesse (YELISETTI et al., 2016), onde são observados resultados promissores quando empregado a privação de soro fetal bovino, inibição por contato e reguladores químicos, tais como a roscovitina (HASHEM et al., 2007). Assim um adequado protocolo de sincronização do ciclo em G₀/G₁ é obtido pelo equilíbrio entre taxa de células em G₀/G₁ e manutenção de sua viabilidade.

Desse modo, é notável a grande variabilidade observada entre diferentes espécies de felídeos durante as etapas de cultivo *in vitro*, criopreservação e sincronização em G₀/G₁ e seu impacto para aplicação das células de cada espécie. Portanto, este estudo comparou diferentes condições de cultivo, criopreservação e sincronização do ciclo celular em fibroblastos de gato-do-mato-pequeno, buscando otimizar protocolos para aplicações biotecnológicas voltadas à conservação da espécie.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. RELEVÂNCIA ECOLÓGICA E DESCRIÇÃO POPULACIONAL DO GATO-DO-MATO-PEQUENO

Os felídeos são importantes carnívoros que podem ser classificados em pequenos e grandes indivíduos de acordo com seu peso, hábitos alimentares e tamanho corporal (MUKHERJEE et al., 2019). Entre os pequenos felídeos, encontra-se o gato-do-mato-pequeno (*L. tigrinus*, **Figura 1**), espécie pertencente ao gênero *Leopardus*, apresentando grandes semelhanças físicas com o gato maracajá (*L. wiedii*) (NASCIMENTO; FEIJÓ, 2017). A espécie pode ser encontrada em locais específicos na América Central, Andes, na região nordeste na América do Sul e nas planícies da Venezuela e Colômbia (BONILLA-SÁNCHEZ et al., 2024).

Quanto aos hábitos do gato-do-mato-pequeno, este se caracteriza como um felídeo com hábitos alimentares noturnos, solitários, sendo, majoritariamente, encontrado em florestas e savanas (OLIVEIRA et al., 2022). Quanto ao seu biótipo, ele tem peso corpóreo entre 1,7 e 3,5 kg e comprimento de aproximadamente 50 cm, semelhante ao gato doméstico (OLIVEIRA-SANTOS et al., 2012). Quando à sua pele, possui uma coloração marrom, podendo ser escura, clara ou acinzentada com manchas arredondadas com bordas pretas e interior marrom escuro (NASCIMENTO; FEIJÓ, 2017).

Nos ecossistemas onde é encontrado, o gato-do-mato-pequeno apresenta um papel fundamental na teia alimentar como mesopredador, influenciando no controle de suas presas e, conseqüentemente, na diversidade da flora (WANG, 2002). Semelhante a outros pequenos felídeos, possui alimentação carnívora, alimentando-se de pequenos roedores, pequenos pássaros e anfíbios, como cobras e lagartos, geralmente presas com peso entre 20 g e 100 g (TORTATO et al., 2021). Devido à sua função como mesopredador, pode ser considerada uma espécie sentinela em virtude de sua interação direta com espécies de nível trófico inferior, sendo diretamente afetada pela diminuição da sua presa (MARNEWECK et al., 2022).

Mesmo com seu importante papel ecológico, tem-se observada uma diminuição na densidade populacional da espécie, em virtude de fatores intrínsecos da natureza ou fatores

extrínsecos. Dentro os fatores intrínsecos, pode-se citar o fato de o gato-do-mato-pequeno ser o menor felídeo das Américas, sendo sua distribuição fortemente influenciada pela competição com felídeos de maior porte (GONZÁLEZ-MAYA et al., 2022). Quanto aos fatores extrínsecos, estes são peças-chaves na redução populacional da espécie, ocasionado em virtude de atropelamentos (SILVA et al., 2022), fragmentação de habitats (ZANIN et al., 2014) e doenças advindas de espécies domésticas (MARINHO et al., 2018).



Figura 1. Exemplar de gato-do-mato-pequeno em confinamento no ECOPOINT Parque Ambiental e Zoológico. Fonte: Figura do autor.

Portanto, devido à importância ecológica da espécie e a redução de seu quantitativo populacional, sendo classificada como espécie vulnerável à extinção (PAYAN; OLIVEIRA, 2016), diversos métodos de conservação têm sido empregados.

2.2. ESTRATÉGIAS DE CONSERVAÇÃO PARA O GATO-DO-MATO-PEQUENO

Dentre as metodologias empregadas para conservação de felídeos, os grandes felídeos, como a onça-pintada, ganham destaque em comparação aos pequenos felídeos, em virtude do

carisma com o público e seu apelo socioeconômico, gerando o aumento da atenção a este grupo em específico (MACDONALD et al., 2022). Segundo MONROY-VILCHIS et al. (2019), apenas 1% dos investimento em conservação tem sido destinado aos pequenos felídeos, ocasionando uma redução do conhecimento sobre as espécies e das estratégias desenvolvidas para sua conservação.

Felizmente, para o gato-do-mato-pequeno, algumas estratégias têm sido realizadas, seja por estratégias *in situ* ou *ex situ*. Dentre aquelas *in situ*, NAGY-REIS et al. (2019) com auxílio de câmeras, realizaram a quantificação populacional, possibilitando definir os hábitos alimentares como generalista e distribuição em florestas tropicais. Além disso, no Brasil por meio do Plano Nacional para conservação de pequenos felídeos são realizadas atividades com intuito de: (1) diminuir as ações antrópicas; (2) conservar a espécie e seu habitat; (3) conscientizar a população por meio da educação ambiental e entendimento da importância da espécie para o ecossistema (SOUZA; BERNARD, 2020).

Em paralelo às estratégias *in situ*, aquelas *ex situ in vivo* tem sido estabelecida, onde por meio da alocação da espécie em zoológicos tem-se desenvolvido estudos para melhoria das suas condições em cativeiro, possibilitando a manutenção das suas características selvagens (RESENDE et al., 2011). Ainda, animais em cativeiro têm sido importantes ferramentas para estudos morfológicos, reprodutivos (MOREIRA et al., 2007), cardiovasculares (CARVALHO et al., 2007), comportamentais (MOTTA; REIS, 2009) e genéticos (FUTAS et al., 2022).

Dentre as estratégias *ex situ* além das realizadas *in vivo*, as metodologias *in vitro* destacam-se em virtude da possibilidade de utilização de amostras de um maior quantitativo de animais, da diversidade genética e por ser um método mais seguro e mais econômico que a manutenção em cativeiro (PROCHOWSKA et al., 2019). Em gato-do-mato-pequeno, protocolos iniciais relacionados às biotecnologias reprodutivas foram desenvolvidos, como o realizado por WIEDEMANN et al. (2013) por meio da criopreservação e cultivo *in vitro* de folículos pré-antrais. Ainda, com gametas, protocolos de coleta por *slicing* foram estabelecidos para obtenção de oócitos derivados de indivíduos *post-mortem* (CARREIRO et al., 2017), coleta de sêmen por meio da eletroejaculação (JUVENAL et al., 2008) e refrigeração de sêmen (ANGRIMANI et al., 2017). Tais estudos demonstram o interesse para realização de metodologias, tais como a produção de embriões, visando o aumento populacional da espécie.

Além dos gametas, amostras somáticas possuem vantagens, como a possibilidade de

recuperação independente do sexo, idade reprodutiva, bem como da capacidade de obtenção de vários tipos celulares de uma mesma amostra (BORGES; PEREIRA, 2019). Com o gato-do-mato-pequeno, Arantes et al. (2021) determinaram as características iniciais das células como morfologia, ultraestrutura, tempo de duplicação e curva de crescimento. Ainda, esses autores realizaram a criopreservação com 2,5% e 10% de dimetilsulfóxido (DMSO), 10% de soro fetal bovino (SFB) e CryoSOfree (um crioprotetor quimicamente definido livre de compostos de origem animal e DMSO), obtendo viabilidade superiores a 80%.

Por outro lado, o estabelecimento de uma linhagem celular se define pela caracterização das células por meio de seu cariótipo e marcadores específicos da superfície celular, assim como pela realização de passagens (RYDER; ONUMA, 2018). Ainda, a criopreservação pode estender seus danos a outras organelas celulares sendo fundamental analisar esses danos ao longo de toda célula. Portanto, estudos mais aprofundados são necessários quanto a esses parâmetros para o gato-do-mato-pequeno.

2.3. BANCOS SOMÁTICOS PARA A CONSERVAÇÃO DE FELÍDEOS SILVESTRES

Os bancos de células somáticas para conservação de felídeos silvestres são de suma importância, uma vez que permitem o armazenamento de muitas amostras, promovendo a diversidade genética (BOLTON et al., 2022), estocagem a longo prazo, possibilitando a aplicação de biotecnologias, como a clonagem por TNCS (YELISETTI et al., 2016) e produção de iPSC (VERMA et al., 2012). Um exemplo da importância e aplicabilidade dos criobancos de células somáticas tem sido o desenvolvimento dos “Frozen Zoo” que alocam amostras biológicas (incluindo células somáticas) em temperaturas criogênicas, visando a realização de estudos relativos à biologia e conservação das espécies (BENIRSCHKE, 1984). No Frozen Zoo, na Califórnia, diferentes amostras estão armazenadas de mais de 550 espécies de mamíferos, tais como leão, guepardo, onça pintada e leopardo (RADIN, 2015). O desenvolvimento desses zoológicos demonstra a importância dos criobancos como método de conservação para espécies silvestres.

Em felídeos silvestres, para geração de criobancos de células somáticas, algumas etapas são fundamentais, tais como: (i) identificação do tipo celular e estabelecimento das condições de cultivo *in vitro*, (ii) determinação das condições de criopreservação de células somáticas, e (ii)

avaliação funcional das células somáticas usando sistemas de sincronização em G₀/G₁ (LIRA et al., 2022). Estes passos têm sido aplicados em diversos estudos de felídeos silvestres (**Quadro 1**).

2.3.1. Caracterização celular e estabelecimento das condições de cultivo *in vitro*

O estabelecimento das condições de cultivo *in vitro* é essencial na formação de bancos de células somáticas, uma vez que a qualidade celular antes da criopreservação depende destas condições estabelecidas. Em geral, células são criopreservadas na terceira passagem, uma vez que o cultivo primário se apresenta heterogêneo, sendo preferível a utilização de cultivo homogêneo (RYDER; ONUMA, 2018). Em cultivos de primeira e segunda passagem, há a presença de tipos celulares com diversos formatos e tamanhos, resultando em variações em seu núcleo e organização celular, afetando seu uso adequado (MANGIAVACCHI et al., 2022; WU et al., 2022).

Inicialmente, tecidos somáticos são obtidos a partir de biópsias de indivíduos *in vivo* ou *post-mortem* (MESTRE-CITRINOVITZ et al., 2016), sendo estabelecido em seguida o cultivo primário. Para a realização dos cultivos secundários, a definição das condições de cultivo é essencial, onde em felídeos tem sido empregado o meio essencial mínimo modificado por Dulbecco (DMEM), adicionado de 10% de SFB com a ausência ou presença de antibióticos (**Quadro 2**). Ainda, em relação as condições atmosféricas têm sido utilizadas 5% de CO₂ e temperaturas de 37,0 a 38,5 °C, promovendo a manutenção da viabilidade celular (MOULAVI et al., 2017; ARANTES et al., 2021).

Adicionalmente, visando a diminuição do tempo necessário para duplicação, suplementos podem ser adicionados durante o cultivo *in vitro*, como fatores de crescimento epidermal (EGF), glicose ou glutamax (**Quadro 2**). Contudo, majoritariamente o meio base com DMEM suplementado com 10% de SFB tem demonstrado ser eficaz no estabelecimento do cultivo *in vitro*. Considerando que a definição de suplementações é um processo laborioso e de alto custo (PARK et al., 2022), ter esse passo já estabelecido é importante para o estudo das outras variáveis no cultivo *in vitro* (PAMIES et al., 2023).

202 **Quadro 1.** Conservação usando congelação lenta de células somáticas em felídeos silvestres.

Espécie	Cultivo <i>in vitro</i>		Solução crioprotetora	Avaliação criopreservação	após	Finalidade	Referência
Gato-da-líbia (<i>Felis silvestris libica</i>)	Estabelecimento linhagem celular	da	DMEM; 10% de DMSO; 10% SFB;	TNCSi;		Obtenção de embriões por TNCSi	GÓMEZ et al. (2004)
Gato-do-deserto (<i>Felis margarita</i>)	Estabelecimento linhagem celular	da	GMEM; 10% de DMSO; 10% SFB;	Apoptose e Necrose; Níveis de acetilação das histonas; TNCSi;	das	Obtenção de embriões por TNCSi	GÓMEZ et al. (2008)
Tigre siberiano (<i>Panthera tigris altaica</i>)	Estabelecimento caracterização linhagem celular	e da	DMEM; 10% DMSO; 50% SFB;	Viabilidade; Curva de crescimento;		Conservação do recurso genético	LIU et al. (2010)
Lince-ibérico (<i>Lynx pardinus</i>)	Estabelecimento linhagem celular	da	DMEM; 5-15% DMSO; 0,1-0,2 M SAC; DMEM/F-12;	Viabilidade; Metabolismo;		Geração de criobanco para espécie	LEÓN-QUINTO et al. (2011)
Guepardo-aisático (<i>Acinonyx jubatus venaticus</i>)	Estabelecimento linhagem celular	da	10% DMSO; 10% SFB; 0,5 Mm PMSF	Viabilidade; TNCSi;		Obtenção de embriões por meio de TNCSi	MOULAVI et al. (2017)
Onça-pintada (<i>Panthera onca</i>)	Estabelecimento caracterização linhagem celular	e da	DMEM 1,5 M DMSO 10% SFB 0,25 M SAC	Viabilidade; Atividade Proliferativa: EROS; Metabolismo; Níveis Apoptóticos;		Geração de recursos genéticos para espécie	SILVA et al. (2021)
Onça-parda (<i>Puma concolor</i>)	Estabelecimento linhagem celular	da	DMEM; 2,5-10% DMSO; 2,5-10% EG; 10% SFB; 0-0,20 M SAC;	Viabilidade; Atividade proliferativa: Metabolismo; Níveis apoptóticos; Morfologia;		Obtenção de criobanco para espécie	RODRIGUES et al. (2022)

203 DMEM: meio mínimo essencial modificado por Dulbecco; DMSO: dimetilsulfóxido; EG: etilenoglicol; GMEM: meio mínimo
 204 essencial modificado por Glasgow; SFB: soro fetal bovino; SAC: sacarose; PMSF: fluoreto de fenilmetilsulfonil.

Em geral, durante o cultivo *in vitro*, a homogeneidade é essencial para aplicação dessas células. Desse modo, diversos estudos realizaram a caracterização celular visando à confirmação do tipo celular, podendo ser realizado por meio da expressão de genes, como observado por Echeverry et al. (2020) com células-tronco de *P. concolor*; imunohistoquímica como realizado por Verma et al. (2012) em células de *Panthera uncia* ou por meio da microscopia eletrônica de varredura como indicado por Arantes et al. (2021) com o gato-do-mato-pequeno.

Além das condições de cultivo *in vitro* ambientais e de meios adequadas, o número de passagens é um fator determinante na caracterização de células somáticas na formação de criobancos, onde deve-se garantir a qualidade dos parâmetros celulares e epigenéticos após um longo número de passagens (LIU et al., 2010). Wittayarat et al. (2013) trabalhando com gato-bravo-dourado-da-Ásia (*Pardofelis temminckii*), gato-marmorado (*Pardofelis marmorata*) e gato-leopardo (*Prionailurus bengalensis*) observaram que células na terceira e sexta passagem não apresentaram diferenças quanto a sua capacidade de sincronização em G₀/G₁. Em contrapartida, células derivadas de onça-parda demonstraram danos em sua morfologia e na sua curva de crescimento na décima passagem (LIRA et al., 2021). Tais estudos têm demonstrado ser fundamentais o entendimento das passagens na qualidade celular ao longo do cultivo *in vitro*.

Finalmente, fibroblastos têm-se demonstrado uma ferramenta importante não somente para o entendimento da célula, mas de todo organismo. Muito tem sido discutido quanto ao uso do fibroblasto como modelo para espécies, uma vez que parte da adaptação do animal e seu ambiente refletem no ambiente *in vitro* (JIMENÉZ; HARPER, 2023). Madelaire et al. (2022) demonstraram que fibroblastos de foca-de-weddell (*Leptonychotes weddellii*), humanos e de rato apresentaram comportamentos distintos ao serem expostos a 4, 25 e 37° C, o que reflete nas condições de cultivo. Portanto, o estabelecimento de uma linhagem celular para gato-do-mato-pequeno proporcionaria melhor compreensão da espécie, hábitos e condições de cultivo *in vitro*.

2.3.2. Determinação das condições de criopreservação de células somáticas

Para o armazenamento das células em criobanco, as condições de criopreservação devem ser ideais para reduzir a formação dos cristais de gelo e garantir o melhor desempenho das células após a descongelação. Em células somáticas, a congelação lenta tem demonstrado ser a metodologia mais adequada, ocorrendo a redução gradual de 1 °C/min, resultando na desidratação lenta da célula (OLIVEIRA et al., 2021).

236 **Quadro 2.** Condições de cultivo *in vitro* de células somáticas de felídeos silvestres.

Espécie	Condições de Cultivo	Meio de Cultivo	de	Número de passagens	Avaliações	Efeito do número de passagens	Referência
Tigre Siberiano (<i>P. tigris altaica</i>)	37°C 5% CO ₂	DMEM; 10% SFB; 100 U/ml penicilina, estreptomicina e anfotericina;		3º passagem	Morfologia; Viabilidade; Curva de Crescimento; Análise Cromossômica; Caracterização;	Sem alterações morfológicas ao longo das passagens, com a obtenção de cultivo homogêneo na 3º passagem.	LIU et al. (2010)
Tigre-de-bengala (<i>P. tigris tigris</i>)	38,5°C 5% CO ₂	DMEM; 10% SFB;		2º a 3º passagem	Morfologia; Viabilidade; Curva de Crescimento; Cariotipagem; Contaminação; Expressão de proteínas;	O número de passagens não alterou nenhum dos parâmetros analisados.	GUAN et al. (2010)
Lince-ibérico (<i>Lynx pardinus</i>)	37°C 5% CO ₂	DMEM; 15% SFB; 4,5 g/L glicose; 10 ng/mL EGF; 25 mM HEPES; 1% de aminoácidos não-essenciais; 0,1 mM 2-mercaptopetanol; 1 mM piruvato de sódio; 100 U/ml penicilina 0,1 mg/mL estreptomicina;		3º passagem	Morfologia; Tempo de duplicação; Viabilidade;	Sem alterações ao longo das passagens.	LEÓN-QUINTO et al. (2011)
Onça-pintada (<i>Panthera onca</i>)	38,5°C 5% CO ₂	DMEM; 10% SFB; 2% ATB/ATM;		4º passagem	Morfologia; Viabilidade; Atividade Metabólica;	Número de passagens não afetou os parâmetros	OLIVEIRA et al. (2021)

Gato-pescador (<i>Prionailurus viverrinus</i>)	38°C 5% CO ₂	DMEM; 20% SFB; 500 μL aminoácidos não-essenciais; 500 μL Glutamax; 500 μL ATB; 500 μL ATM;	4° a 5° passagem	Morfologia; Teste de senescência; Atividade Metabólica; Imunofluorescência; Reprogramação; Alcalina Fosfatase;	morfológicos. Células apresentaram danos, com mudanças morfológicas e aumento da senescência.	SUKPARANGSI et al. (2022)
Jaguarundi (<i>Puma yagouaroundi</i>)	37°C 5% CO ₂	DMEM; 10% SFB;	3° a 4° passagem	Morfologia; Viabilidade; Sincronização do ciclo celular;	Não informado	MŁODAWSKA et al. (2022)
Gato-de-pallas (<i>Otocolobus manul</i>)				Morfologia; Atividade Metabólica; Atividade Proliferativa;	Células na 10° passagem tiveram mudanças morfológicas, ultraestruturais e em sua curva de crescimento.	
Onça-parda (<i>Puma concolor</i>)	38,5°C 5% CO ₂	DMEM; 10% SFB; 2% ATB/ATM;	1° a 10° passagem	Viabilidade; Níveis EROS; Níveis Apoptóticos; MEV; Imunofluorescência;		LIRA et al. (2022)

237 DMEM: meio mínimo essencial modificado por Dulbecco; MEM: meio mínimo essencial modificado; ATB- antibiótico; ATM-
238 antimicóticos; EGF: fator de crescimento epidermal; EROS: espécies reativas de oxigênio; MEV: microscopia eletrônica de varredura;

Além disso, a presença dos crioprotetores auxiliam na manutenção do equilíbrio durante a redução da temperatura e reduzem a formação dos cristais de gelo (FREITAS-RIBEIRO et al., 2022). Quanto à combinação de crioprotetores utilizados em felídeos silvestres, esta consiste na combinação de DMSO em concentrações entre 2,5% a 15%, adicionado de SFB a 10%, majoritariamente, e acrescido ou não de sacarose a 0,1 até 0,25 M (**Quadro 1**). Em gato-do-mato-pequeno, Arantes et al. (2021) obtiveram resultados positivos, promovendo a manutenção da viabilidade celular próxima a 90% com a solução composta por 2,5% ou 10% de DMSO e 10% de SFB. Alternativamente, o etilenoglicol tem tido papel importante como alternativa ao DMSO, sendo considerado com menor toxicidade (POGOZHYKH et al., 2020). Em felídeos, sua eficiência foi observada por Rodrigues et al. (2022) nas concentrações de 2,5% e 10% na presença ou ausência de sacarose com viabilidade e atividade metabólica superior a 78% de células de onça-parda. Contudo, a maioria dos estudos em felídeos ainda emprega o DMSO como intracelular.

Além do SFB, outras soluções extracelulares são utilizadas visando reduzir o efeito citotóxico dos crioprotetores intracelulares, tais como a sacarose, onde em lince-ibérico (*Lynx pardinus*) nas concentrações de 0,1 M ou 0,2 M de sacarose, houve uma taxa de viabilidade superior a 80% (LEÓN-QUINTO et al., 2011, 2014). Além da sacarose, crioprotetores derivados de fontes naturais sem efeito tóxico, tais como a L-prolina, têm sido empregados (JESUS et al., 2022). Quanto a sua eficiência, a L-prolina demonstrou resultados positivos na concentração de 100 mM de células neuronais de rato promovendo viabilidade superior a 50% com a presença de trealose (BAILEY et al., 2015). Portanto, torna-se interessante o estudo de soluções crioprotetoras extracelulares alternativas para o gato-do-mato-pequeno, visando analisar sua ação em distintos parâmetros celulares.

2.3.3. Avaliação funcional das células somáticas usando sistemas de sincronização em G₀/G₁

A sincronização do ciclo celular tem sido referenciada como uma técnica importante, sendo essencial para obtenção de iPSC, promovendo a homogeneização do cultivo, melhorando os métodos de infecção viral (CHEN et al., 2012) ou estimulando a transcrição de DNA recombinante, auxiliando, desta forma, na indução à pluripotência (ZHAO et al., 2016). Ainda, resultados positivos têm sido obtidos com células sincronizadas em G₀/G₁, estando relacionado à coordenação do ciclo celular do carioplasto (célula somática) e do citoplasto (oócito)

(MROWIEC; BUGNO-PONIEWIERSKA, 2022).

Especificamente em felídeos silvestres, as metodologias de sincronização têm demonstrado resultados variados. Nos estudos de Młodawska et al. (2022) com jaguarundi, gato-de-pallas e gato doméstico, os métodos de privação de SFB e inibição por contato por 1 a 3 dias, variaram seus resultados entre 80% e 97% de células sincronizadas, onde a variação foi correlacionada à confluência, a espécie, o tempo e o tipo de tratamento, tornando-se interessante o desenvolvimento de pesquisas que analisem tais parâmetros para sucesso da técnica na espécie de interesse.

Quanto aos tipos de tratamento, a inibição por contato e privação de SFB promovem estado quiescente por meio do aumento da proteína p21 e diminuição da quinase dependente de ciclina (CDK), parando o ciclo celular mediado pelo estresse (PACK et al., 2019). Yelisetti et al. (2016) obtiveram mais de 80% das células de tigre, leão e gato-leopardo sincronizadas após inibição por contato (>90% confluência) e com privação de SFB, possibilitando a obtenção de blastocistos por meio de TNCSi. Quanto ao tempo de tratamento células de gato-chileno (*Leopardus guigna*), células foram tratadas por inibição por contato e privação de soro por 1, 3 e 5 dias, ocasionando aumento de células sincronizadas e com viabilidade superior a 70% (VERAGUAS et al., 2017).

Além disso, inibidores químicos, como a roscovitina que atua diretamente inibindo a CDK, proteínas responsáveis pela diferenciação e transcrição celular (VIDELA-RICHARDSON et al., 2019) tem sido empregado. Em gato-bravo-dourado-da-Ásia (*P. temminckii*) e gato siamês (*F. Catus*), o tratamento com roscovitina em concentrações superiores a 7,5 μ M, 15 μ M e 30 μ M por 24 h, promoveu mais de 80% de células sincronizadas em G₀/G₁ e baixa porcentagem de células em apoptose (WITTAYARAT et al., 2013). Ainda, Selokar et al. (2012) demonstraram que o aumento da concentração da roscovitina pode ocasionar diminuição da viabilidade celular e o aumento de células sincronizadas, sendo de suma importância a definição da concentração adequada a ser utilizada.

3. JUSTIFICATIVA

O gato-do-mato-pequeno é um pequeno felídeo sul-americano de grande importância ecológica, possuindo função como mesopredador, auxiliando no controle de suas presas, e espécie sentinela. Infelizmente, observa-se uma redução populacional na espécie em virtude dos atropelamentos, predação por felídeos maiores e doenças advindas de espécies domésticas. Diante deste cenário, estratégias de conservação *in situ* e *ex situ* tanto *in vivo* quanto *in vitro* têm sido estabelecidas.

Dentre as estratégias *ex situ in vitro*, os primeiros passos têm sido realizados quanto ao estabelecimento de criobancos e caracterização de células somáticas da espécie. Contudo, ao se analisar o panorama destas biotecnologias de conservação três etapas são fundamentais para células somáticas: (i) determinação das condições de cultivo; (ii) determinação do efeito das soluções crioprotetoras e (iii) avaliação da capacidade de sincronização em G_0/G_1 .

Portanto, a proposta teve como objetivo estabelecer as condições de cultivo *in vitro*, criopreservação e sincronização em G_0/G_1 de células somáticas de gato-do-mato-pequeno, possibilitando o desenvolvimento de criobanco para a espécie, bem como a utilização de suas células para a clonagem por TNCS e obtenção de iPSC.

4. HIPÓTESES

I – Células de gato-do-mato-pequeno podem ser cultivadas por até dez passagens sem alterações morfológicas, proliferativas e metabólicas.

II – A adição de sacarose e L-prolina na solução de criopreservação com 2,5% de DMSO promove a manutenção da atividade metabólica, atividade proliferativa e níveis intracelulares de espécies reativas de oxigênio de células somáticas de gato-do-mato-pequeno.

III – O método de inibição por contato por 72 h promove uma maior taxa de células do gato-do-mato-pequeno sincronizadas em G_0/G_1 do ciclo em comparação aos demais métodos de sincronização.

5. OBJETIVOS

5.1. OBJETIVO GERAL

Comparar o número de passagens, crioprotetores e métodos de sincronização em G₀/G₁ de fibroblastos de gato-do-mato-pequeno.

5.2. OBJETIVOS ESPECÍFICOS

- Estabelecer e caracterizar linhagens de células somáticas derivadas da pele auricular de gato-do-mato-pequeno.

- Avaliar os efeitos do número de passagens (3°, 7° e 11°) sobre a viabilidade, atividade proliferativa, metabolismo, análise cromossômica e apoptose de células isoladas do gato-do-mato-pequeno;

- Avaliar o efeito da adição de crioprotetores extracelulares (sacarose e L-prolina) na solução de criopreservação de células somáticas de gato-do-mato-pequeno;

- Determinar o melhor método de sincronização (inibição por contato vs privação de soro fetal bovino vs roscovitina) de células somáticas do gato-do-mato-pequeno de acordo com as taxas de G₀/G₁ e percentual de células viáveis.

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**CAPÍTULO 2- ESTABLISHMENT AND CHARACTERIZATION OF FIBROBLAST
LINES FROM THE NORTHERN TIGER CAT (*Leopardus tigrinus*, SCHREBER, 1775)
DURING EXTENDED PASSAGE AND CRYOPRESERVATION**

Artigo experimental: Establishment and characterization of fibroblast lines from the northern
tiger cat (*Leopardus tigrinus*, schreber, 1775) during extended passage and cryopreservation

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Establishment and characterization of fibroblast lines from the northern tiger cat (*Leopardus tigrinus*, Schreber, 1775) during extended passage and cryopreservation

Running title: Establishment and characterization of northern tiger cat fibroblast line

João Vitor da Silva Viana^a, Lhara Ricarliany Medeiros de Oliveira^a, Luanna Lorena Vieira Rodrigues^a, Yara Letícia Frutuoso e Silva^a, Ana Livia Rocha Rodrigues^a, Alexandre Rodrigues Silva^b, Patrícia Vasconcelos Alves^c, Herlon Victor Rodrigues Silva^d, Alexsandra Fernandes Pereira^{a*}

^aLaboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoro, RN, Brazil.

^bLaboratory of Animal Germplasm Conservation, UFERSA, Mossoro, RN, Brazil.

^cSergento Prata City Zoo, Fortaleza, CE, Brazil.

^dLaboratory of Carnivore Reproduction, State University of Ceará, Fortaleza, CE, Brazil.

*Corresponding author:

Alexsandra Fernandes Pereira

Laboratory of Animal Biotechnology

Federal Rural University of Semi-Arid

Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil

Phone: +55 84 3317 8361

809 E-mail address: alexsandra.pereira@ufersa.edu.br

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811 **Abbreviations**

812 ARTs-Artificial reproductive technologies

813 BSA-Bovine serum albumin

814 CEUA-Ethics Committee of Animal Use

815 DMEM-Dulbecco's modified essential medium

816 DMSO-Dimethyl sulfoxide

817 FBS-Fetal bovine serum

818 H₂DCFDA-2',7'-dichlorodihydrofluorescein diacetate

819 ICMBio-Chico Mendes Institute for Biodiversity Conservation

820 iPSC-Induced pluripotency stem cells

821 MTT-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

822 PBS-Phosphate-buffered saline

823 PDT-Population double time

824 ROS-Reactive oxygen species

825 SCNT-Somatic cell nuclear transfer

826 $\Delta\Psi_m$ -Mitochondrial membrane potential

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828 **Abstract**

829 The establishment of fibroblast lines enables several applications from the formation of biobanks
 830 for the conservation of biodiversity to the use of these cells in physiological and toxicological
 831 assays. Considered a species vulnerable to extinction, the characterization of fibroblastic lines of

northern tiger cat would contribute to its conservation. Therefore, we established and characterized fibroblasts derived from northern tiger cat during extended passage (third, seventh, and eleventh passages) and cryopreservation with regard to the morphology, viability, apoptotic classification, metabolism, proliferative activity, and oxidative stress by reactive oxygen species (ROS) levels and mitochondrial membrane potential ($\Delta\Psi_m$). Initially, we identified four dermal fibroblast lines by morphology, immunophenotyping, and karyotyping assays. *In vitro* culture after the third, seventh, and eleventh passages did not affect the viability, apoptotic classification, and ROS levels. Nevertheless, cells at seventh and eleventh passages featured a reduction in metabolism and an alteration in $\Delta\Psi_m$ when compared to third passage cells. Additionally, cells at eleventh passage showed changes in the proliferative activity and morphology when compared to other passages. Regarding cryopreservation, no effect was observed on cryopreserved cells for morphology, viability, apoptotic classification, metabolism, and proliferative activity. Nevertheless, cryopreserved cells had alteration for ROS levels and $\Delta\Psi_m$. In summary, fibroblasts from northern tiger cat were affected by extended passage (seventh and eleventh passages) and cryopreservation. Adjustments to the *in vitro* culture and cryopreservation are necessary to reduce cellular oxidative stress caused by *in vitro* conditions.

Keywords: Cell line, cryobank, *in vitro* culture, senescence, small wild felids.

1. INTRODUCTION

The northern tiger cat (*Leopardus tigrinus*, Schreber, 1775) is a small wild cat found in South and Central America, in countries such as Argentina, Venezuela, Paraguay, Costa Rica, and Brazil (Jarretta et al., 2004). This species plays a fundamental role in the food chain as a mesopredator, influencing the control of its prey and, consequently, the diversity of the flora (Alberti et al., 2023). Moreover, it has been studied due to its scarce information (Oliveira et al., 2020), lower population density caused by the presence of large felids (Fox-Rosales & Oliveira, 2023), and classification as vulnerable to extinction (Payan & Oliveira, 2016).

Given this scenario, several conservation strategies have been implemented for the northern tiger cat with a focus on *in situ* (Payne et al., 2024), *ex situ in vivo* (Futas et al., 2022), and *ex situ in vitro* (Angrimani et al., 2017) methodologies. In this last group, protocols were developed for sperm recovery (Juvenal et al., 2008), and cryopreservation (Baudi et al., 2007), ovarian tissue *in*

vitro culture and cryopreservation (Wiedeman et al., 2013), oocyte recovery (Carreiro et al., 2017), and somatic cell cryopreservation (Arantes et al., 2021), aiming at the formation of cryobanks. With respect to somatic cells, Arantes et al. (2021) were pioneers in the isolation and cryopreservation of somatic cells from these animals, evaluating different slow freezing conditions. Although the results are promising, the authors suggest optimizing these conditions for adequate conditioning of these cells.

The optimization of these protocols depends on the establishment of cell lines through identification of type and genetic variations (Liu et al., 2010), cell characterization during extended passage (Silva et al., 2021), and assessment of damage caused by cryopreservation (Arantes et al., 2021). In wild felids, skin-derived fibroblasts have been the cells of choice for conservation from these biobanks aiming at nuclear reprogramming (Verma et al., 2012). In this sense, an important point is the cellular senescence, which has been variable, being observed at the fourth passage for the fishing cat (*Prionailurus viverrinus*, Sukparangsi et al., 2022), or tenth passage for the puma (*Puma concolor*, Lira et al., 2022). Additionally, the evaluation of the cell passages is a tool for understanding not just the effect on the reprogramming but also the supplementation of medium (Sukparangsi et al., 2022).

Moreover, associated with the number of passages, the assessment of the damage caused by cryopreservation allows understanding the cellular behavior in the face of stressful conditions (Lira et al., 2022). In general, the cryopreservation can cause physical and biochemical damage on cell metabolism (Murray & Gibson, 2022). Therefore, the evaluation of different cellular quality parameters permits knowing more precisely the cellular response to these conditions (Baust et al., 2017).

Finally, the application of artificial reproductive technologies (ARTs) is directly dependent on the first steps of establishment and characterization of cell lines, and the effects of the cryopreservation over several steps (Thongphakdee et al., 2020). Therefore, we aimed to establish and characterize fibroblasts derived from northern tiger cat during extended passage (third, seventh, and eleventh passages) and cryopreservation with regard to the morphology, viability, apoptotic classification, metabolism, proliferative activity, and oxidative stress. Additionally, we expected to understand and establish the minimum conditions for the formation of cryobanks for the species, contributing to conservation.

2. MATERIALS AND METHODS

2.1. Chemical and media

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and antibiotic-antimycotic solution were obtained from Gibco-BRL (Carlsbad, CA, USA). The anti-vimentin antibody and goat anti-mouse IgG (Alexa Fluor® 488) were purchased from Abcam (Cambridge, MA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Animals care and skin collection

All the experiments were approved by the Ethics Committee of Animal Use of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 20/2023) and the Chico Mendes Institute for Biodiversity Conservation (no. 88201-1; ICMBio). Three males and one female with ages between 2–3 years, located in zoos in cities of Ceara, Brazil, were used for skin recovery.

Ear skin samples (1.0–1.5 cm²) were collected using surgical scissors after administration of 0.04 mg/kg dexmedetomidine (Dexdormitor®, Zoetis, Campinas, SP, Brazil) combined with 5 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, São Paulo, SP, Brazil) and mechanical containment (Juvenal et al., 2008). The tissues were transported to the laboratory in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution at 4 °C for 4–5 h.

2.3. Establishment of primary culture, cell isolation and experimental design

In the laboratory, fragments were trichotomized, fragmented in 6.0 mm³ (3 × 2 × 1 mm), and washed according to the sequence of media: (i) DMEM supplemented with 10% FBS and 10% antibiotic-antimycotic solution; (ii) 70% alcohol, and (iii) DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution (Lira et al., 2022). Subsequently, all fragments (four or five per dish per animal) were cultured in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution at 38.5 °C and 6.5% CO₂.

Monitoring of the primary culture occurred daily using an inverted microscope (Leipzig IMx 400, PhoenixOptics, Germany) with total change of medium. Tissues were evaluated for day

and percentage of attachment, detachment, and subconfluence, duration of culture, and absence of bacteria and fungi. When the cells reached 70% confluence, tissues were removed and cells were trypsinized with 0.25% trypsin-EDTA solution for the first passage (Lira et al., 2022).

The study was divided into three experiments. In the first experiment, cells were grown under primary culture conditions, and when cells reached the third passage were analyzed regarding morphology, confirming the cell type using immunofluorescence assay, karyotyping, and contamination absence. In the second experiment, cells were cultured and evaluated after the third, seventh, and eleventh passages with respect to the effects of the culture on the morphology, viability, apoptotic classification, metabolism, proliferative activity, and oxidative stress by reactive oxygen species (ROS) levels, and mitochondrial membrane potential ($\Delta\Psi_m$). In the third experiment, cryopreserved cells were evaluated using the aforementioned parameters and ultrastructure. The characteristics of the cryopreserved cells were compared with those of non-cryopreserved cells.

2.4. Morphology and immunofluorescence for cell type confirmation

Initially, cells were evaluated for morphological characteristics by observation of cytoplasmatic extensions, shape and cell surface using inverted microscope (Gómez et al., 2008). For immunofluorescence, cells were fixed with 4% paraformaldehyde for 15 min and, subsequently, washed with chilled phosphate-buffered saline (PBS). Cells were incubated with antigen retrieval (100 mM Tris, 5% urea, pH 9.5), permeabilized for 1 h with 0.4% Triton X-100, and incubated with blocking solution (PBS with 5% BSA and 0.1% Tween 20, Silva et al., 2021). Finally, an immunoassay was carried out for 24 h at 4 °C with mouse anti-vimentin antibody (ab8979, 1:200) and then cells were incubated with the secondary antibody (goat anti-mouse IgG, Alexa Fluor® 488, ab150113, 1:400) for 1 h at 25 °C in the dark. The cell nucleus was identified by the incubation with 1 µg/mL Hoechst 33342 for 1 min; the cytoplasmatic extensions were observed in a green fluorescence and the nucleus in a blue fluorescence using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan), with 20× magnification.

2.5. Karyotype analysis

Cells were evaluated by karyotype according to Song et al. (2007), with modifications. Cells with 70% confluence were incubated with 0.1% colchicine for 6 h at 38.5 °C and 6.5% CO₂ for the metaphase synchronization. After this period, cells were trypanized, the pellet was resuspended in 5 mL hypotonic solution (0.075 M KCl), maintained for 10 min at 37 °C and centrifuged (600 × g for 10 min) after incubation. Cells with ruptured membrane and exposed chromosomes were fixed in 5 mL methanol and acetic acid solution, followed by centrifugation (600 × g for 10 min). For the visualization of chromosome structure, fixed chromosome solution was dropped onto slides, dried at 25 °C and stained for 5 min with 5% Giemsa solution. The Giemsa solution excess was washed with distilled water and the slides were dried for visualization at 100× magnification on light microscope (Olympus BX51TF, Tokyo, Japan) and chromosome analysis. The final image of the chromosomes with the optimal staining and definition was selected. The karyotype was arranged based on the Eroğlu (2017) description, using the software GNU Image Manipulation Program (GIMP) version 2.10.22.

2.6. Evaluation of contamination absence

The absence of contamination was assessed by incubating 1.0 mL cell suspension in a 35 × 10 mm well-dish containing a nutrient medium (DMEM with 10% FBS) devoid of antibiotic-antimycotic solution (Lira et al., 2022). The medium was replaced daily, and the cells were examined under an inverted microscope to detect any bacterial and fungal contamination. Additionally, the medium color and opacity were monitored for any changes.

2.7. Cell cryopreservation

Cells at the third passage were trypanized, counted and allocated in cryovials (1.0×10^5 cells/mL) with DMEM supplemented with 10% FBS and 2.5% dimethyl sulfoxide (DMSO) (Arantes et al., 2021). Cells were exposed to cryopreservation solution, allocated in Mr. Frosty freezing container for 15 min at 4 °C. Subsequently, cells were stored overnight at -80 °C freezer with a cooling rate of 1 °C/min. Finally, cells were stored in liquid nitrogen (-196 °C).

After two weeks, cells were thawed in a graduate temperature elevation with exposition for 1 min at 25 °C and immersion in water bath for 4 min at 37 °C. The removal of cryoprotectants was performed twice through dilution with DMEM associated with 10% FBS

followed by centrifugation ($600 \times g$ for 10 min, Rodrigues et al., 2023). Finally, cells were kept for 15 min at 25 °C and cultured in dish for the analysis. Cryopreserved cells were evaluated for the cell quality parameters measures and ultrastructure. The characteristics of the cryopreserved cells were compared with those of non-cryopreserved cells. Additionally, cryopreserved cells were evaluated for viability immediately after thawing and *in vitro* culture.

2.8. Evaluations of cell quality

The cell morphology was analyzed daily using an inverted microscope (Viana et al., 2023) for the observation of cytoplasmatic extensions, colony formation, cell detachment, and confluence. For the viability assay, cells were stained with trypan blue solution (0.4% in PBS) at 1:1 ratio (Rodrigues et al., 2023). Cell numbers were conducted using a Neubauer chamber, where stained cells with ruptured membranes were quantified as non-viable, while unstained cells with intact membranes were considered viable. The viability was determined by viable cells/total cell counted $\times 100$.

Apoptosis levels were determined using acridine orange (2.0 $\mu\text{g/mL}$) and ethidium bromide (10 $\mu\text{g/mL}$, Lira et al., 2022). After incubation, cells were examined under a fluorescence microscope, and the acquired images were subjected to analysis using Image J software (National Institutes of Health, NIH, USA) to quantify the fluorescence signal intensity. Cells were classified based on their characteristics: those with a light green nucleus were deemed viable. Cells exhibiting non-uniform aspects were categorized as early apoptosis. Cells displaying bright orange areas were identified as being in late apoptosis. Lastly, cells with a uniformly orange nucleus were classified as necrotic.

Metabolic activity was analyzed through the conversion of (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into formazan crystals. A cell density of 5.0×10^4 cells/mL was cultured in 12-well dishes for five days under conditions of 38.5 °C and 6.5% CO_2 . After incubation, cells were treated with MTT solution (5 mg/mL in DMEM) for 3 h (Silva et al., 2021). Subsequently, formazan crystals were solubilized using DMSO and the absorbance was measured using a spectrophotometer (Shimadzu® UVmini-1240) at a wavelength of 595 nm.

The population doubling capacity analysis and growth curve determination were carried out following the methodology described by Rodrigues et al. (2023). A concentration of 7.0×10^4

cells/mL was evenly distributed across seven wells in a 24-well dish. Each well was trypsinized and quantified every 24 h over a total period of 168 h. The cell concentrations measured at each 24-h interval were utilized to construct the growth curve and calculate the population doubling time (PDT) using the formula: $PDT = (T \times \ln 2) / \ln (X_e / X_b)$, where T represents the incubation time, X_b and X_e , respectively, denote the initial and final cell counts at the beginning and end of the incubation period, and $\ln$ refers to the natural logarithm.

Fluorescence probes, 5 μ M 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and 500 nM MitoTracker Red (CMXRos) were used to assess ROS levels and $\Delta\Psi_m$. Cell suspensions (100 μ L each) were exposed to the respective probe for 30 min under standard cell culture conditions (38.5 $^{\circ}$ C and 6.5% CO_2). Subsequently, cells underwent centrifugation ($300 \times g$ for 3 min), followed by visualization using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). Fluorescence intensity was quantified using Image J software (National Institutes of Health, NIH, USA).

Finally, non-cryopreserved and cryopreserved cells were assessed for ultrastructural damage, focusing on alterations to the cell surface and cytoplasmic extensions. For evaluation, cells were fixed in 4% glutaraldehyde for 12 h, followed by contrast fixation with 1% osmium tetroxide for 1 h, and dehydration through a graded alcohol series (Lira et al., 2022). Cells were metalized and subsequently visualized using a scanning electron microscope (TESCAN VEGA3; Tescan Analytics, Fuveau, Bouches-du-Rhône, France).

2.9. Statistical analysis

A total of four cell lines were used, being one cell line per animal. All the cell lines were submitted to the analysis. Data were expressed as mean $\pm$ standard error, considering one animal/one cell line/ one repetition, and analyzed using GraphPad software (Graph-Pad Software Incorporation, La Jolla, USA). Normality and homoscedasticity were performed in all results using Shapiro-wilk test and Levene's test, respectively. All data were altered with arcsine and analyzed using analysis of variance (ANOVA) followed by the Tukey test or unpaired t-test. Statistical significance was considered when $P < 0.05$.

3. RESULTS

3.1. Cell type confirmation and karyotype analysis

Cell isolation was conducted using 4.3 ± 0.3 explants per animal. Notably, each animal exhibited variability in both the quantification of explant attachment and the subconfluence (Table 1). This variability was further evident across days of attachment and subconfluence. Moreover, this variability persisted throughout subsequent different passages, with cells reaching 70% confluence at the third, seventh, and eleventh passages after 26.5 ± 0.3 , 43 ± 0.6 and 96.7 ± 8.3 days, respectively.

Table 1. Description of primary cell culture of somatic tissues and cells derived from northern tiger cat.

Animal (Sex)	No. samples			Days		
	Initial	Attached (%)	Subconfluence (%)	Attached	Subconfluence	Total cell culture
A1 (M)	5	100 (5/5)	100 (5/5)	4	9	15
A2 (M)	4	75 (3/4)	75 (3/4)	1	8	16
A3 (M)	4	100 (4/4)	100 (4/4)	1	8	17
A4 (F)	4	100 (4/4)	75 (3/4)	3	10	17
Mean± S.E.	4.3 ± 0.3	94.0 ± 6.0	88.0 ± 7.0	2.3 ± 0.8	8.8 ± 0.5	16.5 ± 0.5

M: Male; F: Female; Mean ± Standard Error. $P > 0.05$

The cell type was confirmed through vimentin identification using immunofluorescence assay, where cytoplasmic extensions exhibited strong green fluorescence (Figure 1A), while the nucleus was delineated by blue fluorescence emission (Figure 1B). The merged images facilitated the comprehensive visualization of both the nucleus and the cytoplasmic projections as

fibroblasts (Figure 1C). Karyotype analysis revealed a diploid count of $2n = 36$ chromosomes (Figure 1D), with a fundamental number (FN) of 68. This analysis enabled the identification of 34 autosomal chromosomes and two sex chromosomes in male (Figure 1E), classified as 26 metacentric chromosomes and eight submetacentric chromosomes.

Moreover, cells were cultured without the presence of antibiotic/antimycotic agents, and throughout the 27-day period, no changes in color, viscosity, or turbidity of the medium were observed. Additionally, no significant alterations in cell proliferation were observed. Importantly, no evidence of fungal structures or bacterial contamination was detected during this period.

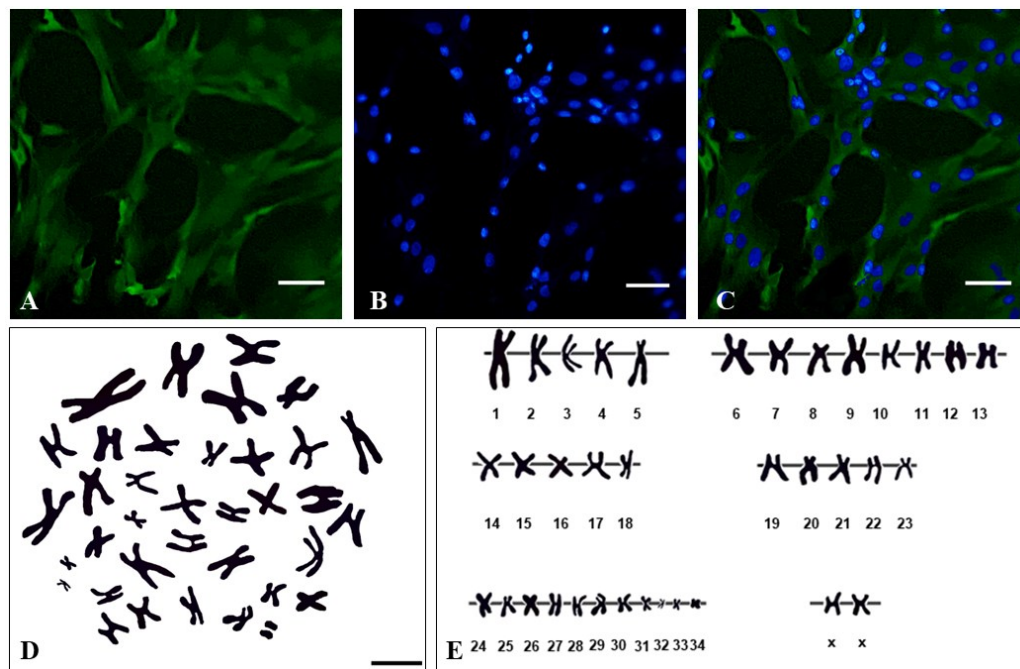


Figure 1. Cell type characterization using immunophenotyping and karyotyping assays in northern tiger cat. (A) Fibroblasts of one animal (A2, male) stained with vimentin (green) identifying the cytoplasmatic projection. (B) The same fibroblasts of animal stained with Hoechst 33342 (blue) identifying the nucleus. Scale bar 50 μm , 40 $\times$. (C) Overlapping stained nucleus and vimentin. (D, E) Karyotype of one animal (A2, male) in the third passage, $n = 36$. Scale bar 100 μm , 100 $\times$.

3.2. Effects of extended passages on culture quality

The primary culture exhibited typical heterogeneity, containing fibroblasts and circular cells (Figure 2A). Fibroblasts, characterized by oval and central nuclei, along with fusiform

morphology and numerous cytoplasmic projections, were observed at the third (Figure 2B) and seventh (Figure 2C) passages. However, morphological alterations, including increased cell size and decreased cytoplasmic projections, were evident at the eleventh passage (Figure 2D). Even with these changes, viability rates remained stable during the passages (Figure 3A), with no differences observed between the third ($91.7\% \pm 4.2$), seventh ($84.5\% \pm 5.8$), and eleventh ($86.2\% \pm 5.9$) passages ($P > 0.05$).

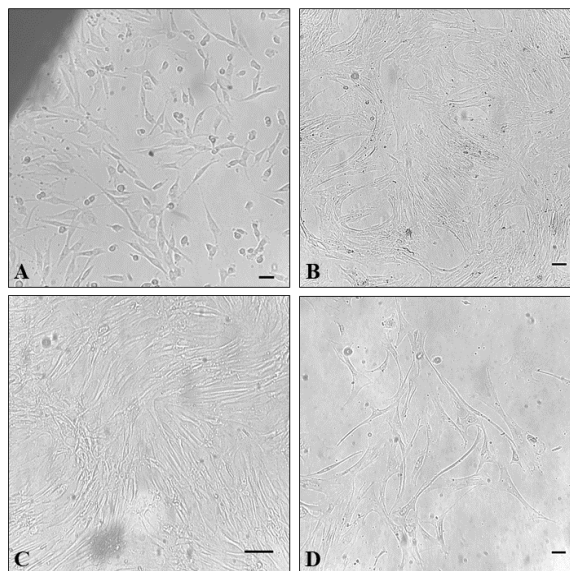


Figure 2. Evaluation of morphology of fibroblasts derived from northern tiger cat after extended cell culture. Fibroblasts during (A) primary cell culture, (B) third, (C) seventh, (D) eleventh passage. Scale bar 50 μm , 20 $\times$.

Contrasting with the viability, metabolic activity decreased progressively from the third ($91.2\% \pm 5.2$) to the seventh ($56.0\% \pm 2.9$) and eleventh ($22.8\% \pm 3.8$) passages (Figure 3B). Interestingly, the apoptotic levels remained unchanged between the passages (Figure 3C, C' and C''), resulting in a high percentage of viable cells observed at the third ($98.7\% \pm 0.4$), seventh ($91.4\% \pm 3.3$), and eleventh ($74.5\% \pm 13.6$) passages, and no increases were noted in early apoptotic, late apoptotic, or necrotic cells (Figure 3D).

Additionally, PDT remained unaffected between the third ($42.5 \text{ h} \pm 4.0$) and seventh ($39.8 \text{ h} \pm 8.3$) passages (Figure 3E). However, at the eleventh passage, only one animal exhibited an adequate cell concentration and was analyzed, revealing an elevated PDT of 85.4 h. The growth curve displayed a sigmoidal shape at both the third and the seventh passages (Figure 3F), characterized by similar exponential phases lasting around two days, followed by stationery and

latency phases. Interestingly, at the eleventh passage, the exponential phase occurred later and was succeeded by a decrease in cell concentration in the subsequent days.

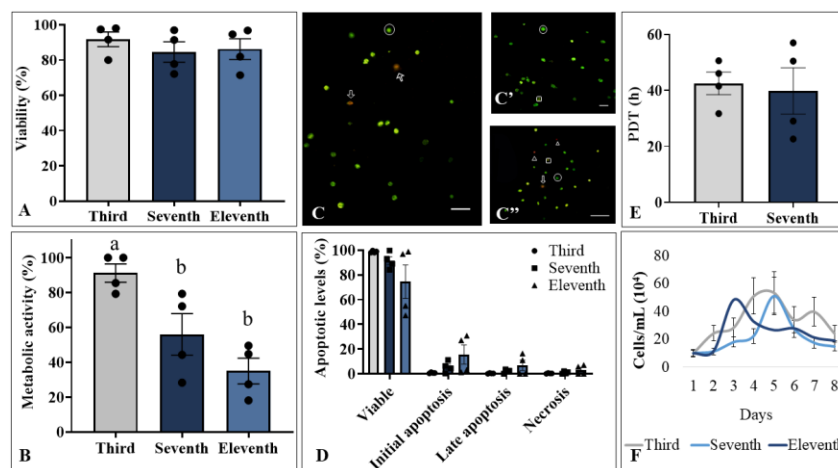


Figure 3. Viability and proliferation of cells derived from northern tiger cat cell after extended cell culture (third, seventh, and eleventh passages). (A) Cell viability by trypan blue assay. (B) Metabolic activity determined by the MTT assay. Apoptosis levels by identification with acridine orange and ethidium bromide after (C) third, viable cells (Circle); Necrotic cells (Arrow), (C') seventh, viable cells (Circle); Initial apoptotic (Square), and (C'') eleventh passage, viable cells (Circle); Initial apoptotic (Square); Later apoptotic (Triangle); Necrotic cells (Arrow). (D) Apoptotic levels quantification. (E) Population double time (PDT) after seven days of cell culture. (F) Growth curve after seven days of cell culture. Scale bars 50 μ m, 40 $\times$ (C, C' and C'').
^{a,b}: Values with different superscript represent significant differences ($P < 0.05$).

Despite the prolonged cell culture duration, ROS levels remained consistent between the third (Figure 4A), seventh (Figure 4B), and eleventh (Figure 4C) passages (1.00 ± 0.73 arbitrary fluorescence units (AFU) vs. 0.87 ± 0.58 AFU vs. 0.92 ± 0.63 AFU, respectively) (Figure 4D). Assessments indicated an increase in $\Delta\Psi_m$ (Figure 4E, F and G) at the seventh (2.33 ± 1.35 AFU) and eleventh (2.35 ± 1.14 AFU) passages compared to the third passage (1.00 ± 0.62 AFU) (Figure 4H).

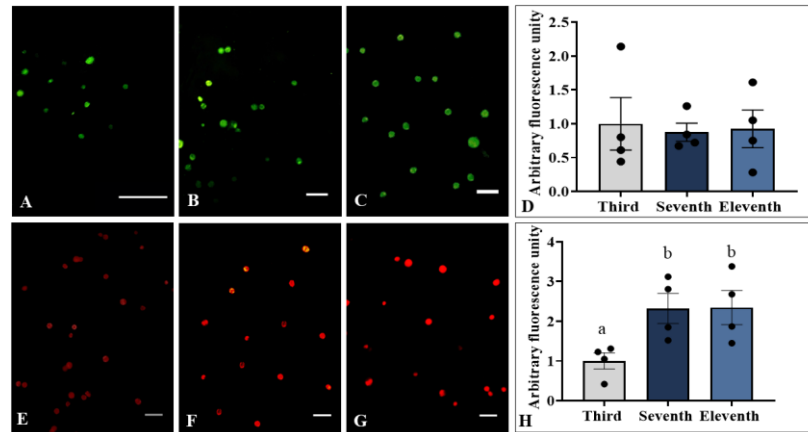


Figure 4. Oxidative stress and potential mitochondrial membrane after third, seventh, and eleventh passages in cells derived from northern tiger cat. Marked cells with H₂DCFDA during (A) third, (B) seventh and (C) eleventh passage. (D) ROS level quantification in arbitrary fluorescence units. Image of potential mitochondrial membrane marked with MitoTracker Red during (E) third, (F) seventh and (G) eleventh passages. (H) Potential mitochondrial membrane using arbitrary fluorescence units measurement; Scale bars 50 μ m, 40 $\times$; ^{a,b}: Values with different superscript represent significant differences ($P < 0.05$).

3.3. Effects of cell cryopreservation on culture quality

Morphological changes were not observed under light microscopy (Figure 5A and 5B), with cells maintaining their fusiform shape, central nucleus, and numerous cytoplasmic projections. However, ultrastructural analysis revealed significant differences between the non-cryopreserved and cryopreserved cells. Non-cryopreserved cells exhibited large size and extensive cytoplasmic projections (Figure 5A'), whereas the cryopreservation led to a reduction in cytoplasmic projections (Figure 5B').

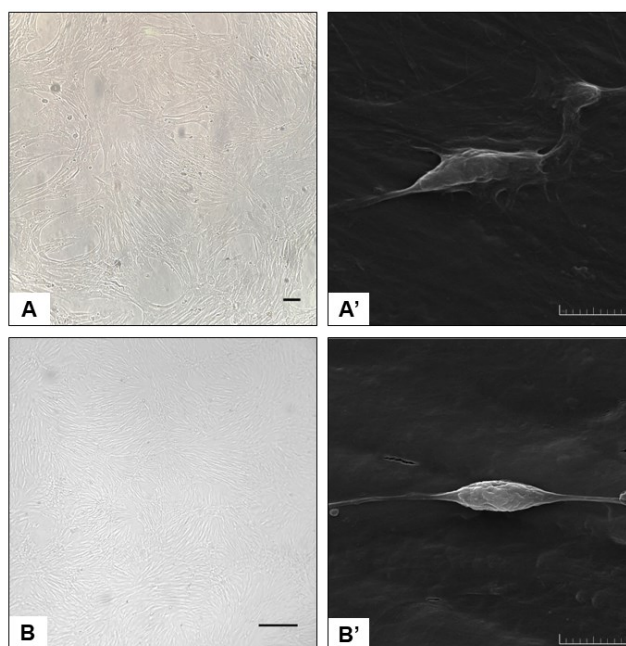


Figure 5. Morphology and ultrastructure of fibroblasts after cryopreservation derived from northern tiger cat. Morphology of (A) non-cryopreserved and (B) cryopreserved fibroblasts under an inverted microscope. Scale bar: 50 μm , 20 $\times$. (A') Ultrastructure of non-cryopreserved and (B') cryopreserved cells with a fusiform shape and cell projections. Scale bar: 5 μm .

Initial post-thawing viability was notably impacted ($64.7\% \pm 3.9$), but *in vitro* culture increased the viability ($95.6\% \pm 1.5$), approaching levels observed in non-cryopreserved cells ($91.7\% \pm 4.2$, Figure 6A). Furthermore, metabolic activity (Figure 6B) was preserved after cryopreservation ($91.2\% \pm 5.2$ vs. $72.7\% \pm 6.5$). Apoptotic levels were unchanged, with viable cells (Figure 6C) comprising $98.7\% \pm 0.4$ pre-cryopreservation and $95.1\% \pm 2.9$ post-cryopreservation, contributing to low rates of early apoptotic (Figure 6D), late apoptotic (Figure 6E), and necrotic cells (Figure 6F), all around 0.3% (Figure 6G).

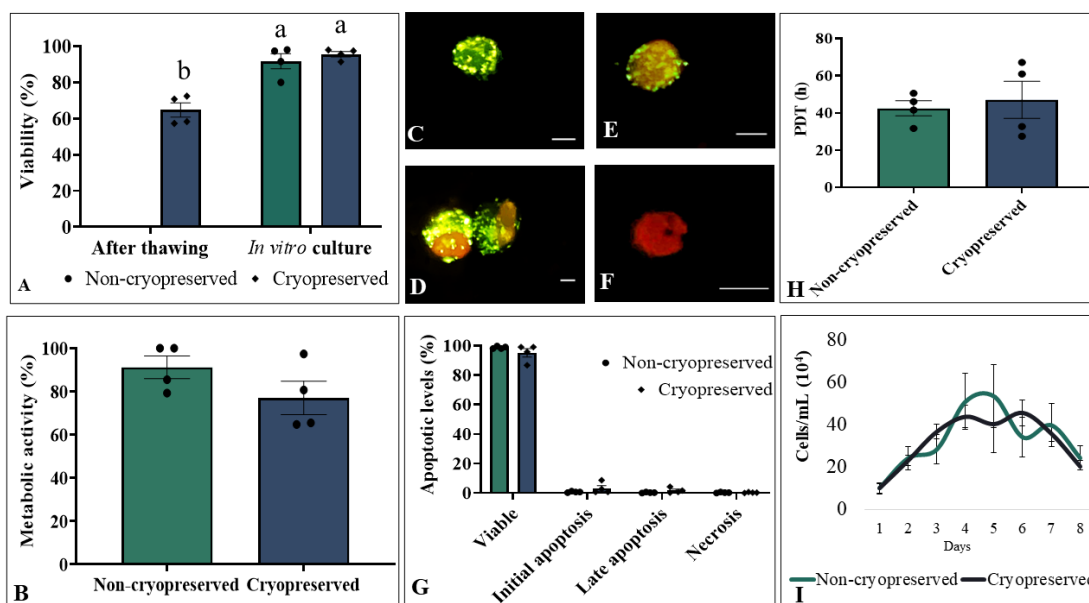


Figure 6. Evaluation of cell parameters of northern tiger cat cells after cryopreservation. (A) Viability measured with trypan blue. (B) Metabolic activity measurement with MTT assay. Cells of third passage marked with acridine orange and ethidium bromide (C) Viable cells, (D) initial apoptotic, (E) late apoptotic (F) necrotic cells. (G) Quantification of apoptosis levels. (H) Growth curve. (I) Population double time; (C, D, E, and F) Scale bars 50 μ m, 100 $\times$. $P > 0.05$.

Parameters of cell division such as PDT (Figure 6H) remained unaffected by cryopreservation (non-cryopreserved cells: $42.5 \text{ h} \pm 4.0$ vs cryopreserved cells: $47.1 \text{ h} \pm 9.9$), and the cell growth curve (Figure 6I) exhibited similar sigmoidal shapes with defined exponential, latency, and stationary phases. Additionally, cryopreservation led to a decrease in ROS levels, how it is observed in the fluorescence before (Figure 7A) and after cryopreservation (Figure 7A'), reducing from 1.00 ± 0.73 to 0.75 ± 0.58 AFU (Fig. 7B). The stress induced by cryopreservation was evident in $\Delta\Psi_m$ after cryopreservation (Figure 7C vs Figure 7C'), which increased from 1.00 ± 0.62 AFU to 3.79 ± 1.61 AFU after cryopreservation (Figure 7D).

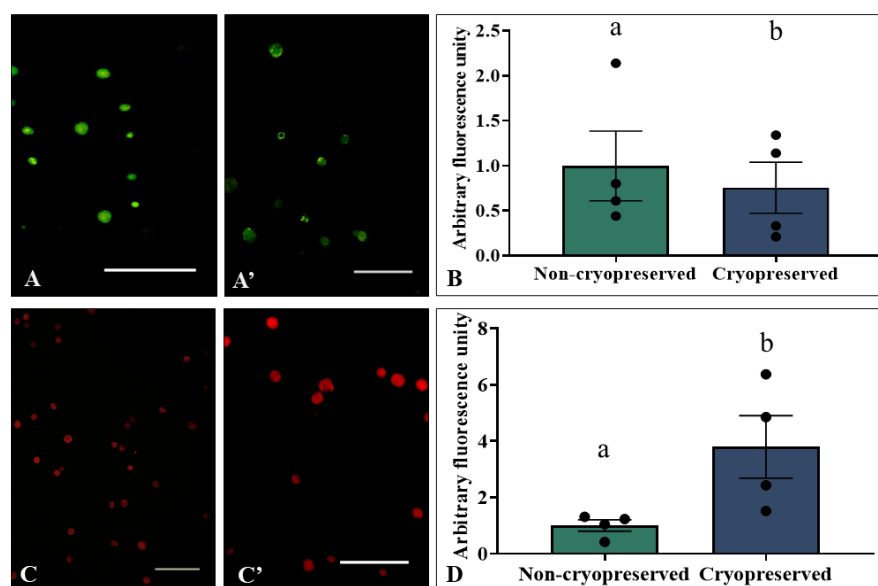


Figure 7. Evaluation of ROS levels by H₂DCFDA and mitochondrial membrane potential with MitoTracker Red after northern tiger cat cells cryopreservation. (A) Non-cryopreserved and (A') cryopreserved cells marked with H₂DCFDA. (B) Measurement of arbitrary fluorescence units for ROS levels quantification; (C) Non-cryopreserved and (C') cryopreserved cells marked with MitoTracker Red. (D) mitochondrial membrane potential evaluation by arbitrary fluorescence unity quantification. Scale bars 50 μ m, 40 $\times$. ^{a,b}: Values with different superscript represent significant differences ($P < 0.05$).

4. DISCUSSION

Arantes et al. (2021) were pioneered in the isolation, ultrastructure characterization, and cryopreservation of somatic cells derived from northern tiger cat. Differently, our study contributes to validate the cell type by immune identification and offers karyotype characterization for the establishment of stable cell lines. Additionally, recognizing the potential stress induced by cell culture and cryopreservation, the study investigated the effects of extended cell passages and cryopreservation.

The confirmation of purity of cell culture was performed at third passage, with vimentin, a filament founded in fibroblasts cytoskeleton. The expression of vimentin makes the cell identification possible and can be an indicative of cell quality biophysics (Sliogeryte & Gavara,

2019). Moreover, the analyzed complete karyotype is an important key for genetic studies and conservation of species. In wild felids, for example, the right accessibility of genetic material has been used to study the evolution of tigers and demographic history (Khan, 2022). Moreover, karyotype analysis had been an essential tool for somatic cell nuclear transfer (SCNT), trying to avoid the number of abnormalities in produced embryos (Veraguas et al., 2020; Song et al., 2007).

The time for subconfluence during the primary culture of northern tiger cat cells was similar, as described for other wild felids, such as ocelot (*Leopardus pardalis*, Viana et al., 2023) and puma (*P. concolor*, Lira et al., 2022), with a mean value of 16 days. Additionally, the primary culture showed a heterogeneity of cells with shapes similar to fibroblasts and keratinocytes. Also, the homogeneity of fibroblasts after three subcultures was similar to the cell line of fishing cat (*Prionailurus viverrinus*, Sukparangsi et al., 2022).

The growth curve of northern tiger cat was described by Arantes et al. (2021) with an increase in cell concentration after 24 h. However, these authors did not calculate PDT precisely. Differently, in our study, PDT was around 40 h, similar to other felids, such as tiger (*Panthera tigris tigris*), with PDT at 34.7 h (Yellisetti et al., 2016), and puma, with PDT at 31 h (Lira et al., 2022). Additionally, cells of northern tiger cat decreased PDT and changed the growth curve during the eleventh passage, similar to puma (Lira et al., 2022) and jaguar (Silva et al., 2021). Madelaire et al. (2022) indicated a specie-specific resistance of cell passages, due to the physiology and natural habits. Apparently, wild felid cells have a sensitivity to *in vitro* stress after the tenth passage, diverging of other species, such as Chinese muntjac (*Muntiacus reevesi*), at the eleventh passage (Wang et al., 2020), or hippopotamus (*Hippopotamus amphibius*), at the twelfth passage (Wang et al., 2021).

The effect of senescence markers was also observed on mitochondrial activity between the seventh and eleventh passages. In general, the senescent cells had a decreased metabolic activity occasioned by DNA damage and lower quantification of NAD^+/NADH (Shmulevich & Krizhanovsky, 2020). Even with damages, cell viability and apoptotic levels were unaltered. Probably, these cells could be in a replicate senescence, where the limitation of *in vitro* cell duplication happens (Kamal et al., 2020).

The damage occasioned to mitochondria was also observed in the increase in $\Delta\Psi\text{m}$ and ROS levels. The increase in $\Delta\Psi\text{m}$ can be explained by the increase in mitochondria mass, and these

modifications can decrease the ROS levels by the increase in electron transport (Miwa et al., 2022). Additionally, the necessity of repairing the cell damage during extended cell culture results in an ATPs necessity, increasing the $\Delta\Psi_m$ levels (Zorova et al., 2018).

Understanding these effects of cell passage on northern tiger cat cells can contribute to the next steps for conservation of species. For wild mammals that can be affected by the prolonged cell culture, the medium supplementation could be optimized, such as in fishing cat (Sukparangsi et al., 2022), or African savanna elephants (*Loxodonta africana*) (Van Vuuren et al., 2023). This information can contribute specially for efficient reprogramming in induced pluripotency stem cells (iPSC, Sukparangsi et al., 2022). Also, the cell line can be applied as an important physiology study tool, aiding to understand differences between species and their adaptability (Pickering et al., 2015; Madelaire et al., 2022).

Moreover, the cryotolerance was an important parameter for the establishment of cell lines, as observed in other species, such as Siberian tiger (*Panthera tigris altaica*, Liu et al., 2010), Iberian lynx (*Lynx pardinus*, León-Quinto et al., 2011), and Bengal tiger (*Panthera tigris tigris*, Guan et al., 2010). Arantes et al. (2021) performed the cryopreservation of somatic cells of northern tiger cat with 2.5% DMSO, with efficiency in the viability and lower number of necrosis cells, which was similar to our study. Additionally, these results were achieved by DMSO being effective in the membrane permeability at low temperature, which can facilitate the cell dehydration during cryopreservation, recovering cells with higher viability (Jungare et al., 2022). In our study, 2.5% DMSO were effective in the proliferative and metabolic activity. However, 2.5% DMSO affected the $\Delta\Psi_m$ and ROS levels of cells of the northern tiger cat. Lower ROS levels can affect the cell equilibrium, and in normal levels these molecules were associate with interaction with phosphatases, kinases, and cell proliferation (Brillo et al., 2021).

Recently, lower exposure concentration of DMSO has been applied due the less toxicity in metabolic activity, enzymes functions, and apoptosis (Erol et al., 2021). On the other hand, in puma cell cryopreservation, the increase in DMSO concentration with 0.2 M sucrose promoted the conservation of the ROS levels. The use of DMSO with extracellular cryoprotectants has showed greater results, once DMSO increases the membrane permeability in extracellular cryoprotectants (Whaley et al., 2021). Probably, adjustments in the use of extracellular cryoprotectants in the small wild cat cell cryopreservation solution could improve conservation efficiency.

In conclusion, fibroblasts from northern tiger cat were affected by extended passage (seventh and eleventh passages) and cryopreservation. Adjustments to the *in vitro* culture and cryopreservation are necessary to reduce cellular oxidative stress caused by *in vitro* conditions. These results described, at first, the cellular characteristics according to different quality parameters of northern tiger cat samples during extended *in vitro* culture and cryopreservation, providing a valuable source for assisted reproduction techniques, aiming at the genetic conservation of this species.

Conflict of interest

The authors declare that they have no conflict of interest.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author (A.F. Pereira).

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Authors contributions

J.V.S. Viana (Investigation, Visualization, Methodology, Writing), L.R.M. Oliveira, L.L.V. Rodrigues, Y.L.F. Silva, A.L.R. Rodrigues (Investigation, Visualization, Methodology); A.R. Silva, P.V. Alves, H.V.R. Silva (Conceptualization, Methodology); A.F. Pereira (Conceptualization, Funding acquisition, Methodology, Supervision, Writing-review & editing).

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**CAPÍTULO 3- NO SYNERGISTIC EFFECT OF EXTRACELLULAR
CRYOPROTECTANTS WITH DIMETHYL SULFOXIDE IN THE CONSERVATION
OF NORTHERN TIGER CAT FIBROBLASTS**

Artigo experimental: No synergistic effect of extracellular cryoprotectants with dimethyl sulfoxide in the conservation of northern tiger cat fibroblasts

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1486 **No synergistic effect of extracellular cryoprotectants with dimethyl sulfoxide in the**
1487 **conservation of northern tiger cat fibroblasts**

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1489 João Vitor da Silva Viana^a, Lhara Ricarliany Medeiros de Oliveira^a, Luanna Lorena Vieira
1490 Rodrigues^a, Yasmin Beatriz França Moura^a, Antonia Beatriz Mendonça Pereira^a, Patrícia
1491 Vasconcelos Alves^b, Herlon Victor Rodrigues Silva^c, Alexsandra Fernandes Pereira^{a*}

1492

1493 ^aLaboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA),
1494 Mossoro, RN, Brazil. ^bSargento Prata Municipal Zoo, Fortaleza, CE, Brazil. ^cLaboratory of
1495 Carnivores Reproduction, University of Ceara State, Fortaleza, CE, Brazil.

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1497

1498 *Corresponding author: Alexsandra Fernandes Pereira

1499 Laboratory of Animal Biotechnology

1500 Federal Rural University of Semi-Arid

1501 Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil

1502 Phone: +55 84 3317 8361

1503 Email address: alexsandra.pereira@ufersa.edu.br

1504

Abstract

The success of somatic cell cryobanks is dependent on establishing reproducible cryopreservation methodologies. We supposed that associated extracellular cryoprotectants (sucrose and L-proline) with 2.5 or 10% dimethyl sulfoxide (Me₂SO) could guarantee better northern tiger cat cells quality rates after thawing when compared to Me₂SO alone. Therefore, we evaluated the effects of sucrose or L-proline with 2.5 or 10% Me₂SO on the cryopreservation of northern tiger cat fibroblasts. Somatic cells were also cryopreserved with 2.5% or 10% Me₂SO alone. All cells were analyzed for morphology, membrane integrity, proliferative activity, metabolism, apoptosis classification, reactive oxygen species (ROS) levels, and mitochondrial membrane potential ($\Delta\Psi_m$). Regardless of the cryoprotective solution, cryopreservation did not affect morphology, membrane integrity after culture, proliferative activity, and metabolism ($P > 0.05$). However, immediately after thawing, 2.5% Me₂SO with L-proline and 10% Me₂SO promoted higher rates of membrane integrity when compared to the other cryopreserved groups ($P < 0.05$). Interestingly, cells cryopreserved with 10% Me₂SO maintained ROS levels similar to non-cryopreserved cells ($P > 0.05$). However, the percentage of viable cells evaluated by apoptosis classification was reduced when using 10% Me₂SO with L-proline compared to non-cryopreserved groups ($P < 0.05$). Additionally, $\Delta\Psi_m$ was altered in all cryopreserved groups ($P < 0.05$). In summary, sucrose and L-proline were less effective in cryopreservation of northern tiger cat fibroblasts in the presence of 2.5% or 10% Me₂SO. Also, 10% Me₂SO appears to be the most suitable cryoprotectant for the formation of cryobanks of this species.

Keywords: biodiversity conservation, cryopreservation, sucrose, L-proline, somatic cell

1. Introduction

The northern tiger cat (*Leopardus tigrinus*, Schreber, 1775) is a small wild felid from Brazil with ecological impact on ecosystem balance [10]. According to the International Union for Conservation of Nature (IUCN) Red List, they are categorized as vulnerable to extinction due to human interference [24]. Given this scenario, conservation strategies have been proposed, such as somatic cell cryobanks [1]. These cryobanks are important tools for the conservation of wild felids [20, 25] and have been created in different regions mainly due to the global concern for maintaining biodiversity [14, 30].

Arantes et al. [1] developed the first study in northern tiger cats for somatic cell cryobanks. The authors observed that 2.5% and 10% dimethyl sulfoxide (Me₂SO) efficiently protected the somatic cells of the northern tiger cats. Although reduced concentrations of Me₂SO emerge as new options for the cryopreservation of somatic cells of wild felids [1, 27], by reducing intracellular cryoprotectant toxicity, we hypothesized that this toxicity could also be reduced with the addition of extracellular cryoprotectants [16, 17]. These extracellular cryoprotectants can prevent apoptosis and osmolarity damage during cryopreservation [3]. Additionally, we observed that systematic studies to evaluate different cryoprotectants in somatic cell preservation in northern tiger cats have been limited.

A new generation of extracellular cryoprotectants has been discovered based on its natural origin [26]. L-proline is an extracellular cryoprotectant that can reduce the formation of large ice crystals [19]. Interestingly, L-proline showed synergism with Me₂SO, increasing the cell viability

and reducing the reactive oxygen species (ROS) levels, especially at lower concentrations of Me₂SO (5% or 7.5%) in human epithelial cells [8]. The effects of L-proline on ice crystal were described at concentrations >100 mM, influencing cell metabolism [3].

Sucrose is also an extracellular cryoprotectant, acting on membrane stability and osmotic cell regulation, and its synergism improves Me₂SO action [7]. In somatic cells of wild felids, 0.2 M sucrose with 10% Me₂SO increased metabolic activity in the Iberian lynx (*Lynx pardinus*) from 50 to 80% [16]. This combination in jaguar (*Panthera onca*) somatic cells conserved the viability and metabolic activity [22]. However, in pumas (*Puma concolor*), sucrose did not increase the viability or metabolic activity of somatic cells [28].

Therefore, since the success of somatic cell banks depends on optimizing the choice of cryoprotectants and on reducing the toxicity of intracellular cryoprotectants, we evaluated the synergic effects of sucrose and L-proline with 2.5 or 10% Me₂SO on the cryopreservation of northern tiger cat fibroblasts.

2. Material and methods

2.1. Chemicals and media

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin solution, and fluorescence probes were obtained from Gibco-BRL (Carlsbad, CA, USA). All the other chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

1570

1571 *2.2. Ethics statement and ear skin collection*

1572 The experimental protocols were approved by the Animal Ethics Committee (CEUA/UFERSA,
1573 no. 20/2023) and the Chico Mendes Institute for Biodiversity Conservation (no. 88201-1;
1574 ICMBio). Ear skin samples (1.0-1.5 cm²) of three males and one female derived from zoos in
1575 Ceará, with ages between 2 to 3 years, were used. All samples were transported at 4 °C for 4-5 h
1576 to the laboratory in DMEM with 10% FBS and 2% penicillin and streptomycin solution.

1577

1578 *2.3. Cell isolation*

1579 The ear skin of each animal was fragmented into 6.0 mm³ (3×2×1 mm) and cultured in DMEM
1580 with 10% FBS, 2% penicillin, streptomycin solution at 38.5 °C, and 6.5% CO₂. Fragments were
1581 cultured until the somatic cells surrounded the tissues. Subsequently, cells were cultured until
1582 70% confluence and submitted to the first passage. Cells were cryopreserved using different
1583 cryopreservation solutions after the third passage, being four days for each passage, and after
1584 characterization as fibroblasts [12].

1585

1586 *2.4. Experimental design*

1587 Fibroblasts were cryopreserved in six solutions containing DMEM, 10% FBS, and (i) 2.5%
1588 Me₂SO, (ii) 2.5% Me₂SO with 0.2 M sucrose, (iii) 2.5% Me₂SO with 100 mM L-proline, (iv)
1589 10% Me₂SO, (v) 10% Me₂SO with 0.2 M sucrose, and (vi) 10% Me₂SO with 100 mM L-proline.
1590 Cells not subjected to cryopreservation were used as controls. All cells were analyzed for

morphology, membrane integrity, proliferative activity, metabolism, apoptosis classification, ROS levels, and mitochondrial membrane potential ($\Delta\Psi_m$).

2.5. Fibroblast cryopreservation by slow freezing

The cryopreservation was performed as described by Rodrigues et al. [28]. A 1.0×10^5 cell/mL concentration was allocated in cryovials and exposed to the cryopreservation solution only with Me₂SO for 15 min at 4 °C. For the solution with sucrose or L-proline, cells were exposed for an additional 15 min at 4 °C. All cryovials were transferred to a freezing container (Mr. Frosty, Thermo Scientific Nalgene, Rochester, NY, USA), and the container was placed in a -80 °C freezer (1 °C/min) cooling rate until -80 °C. Then the cryovials were plunged into liquid nitrogen (-196 °C) for storage.

After two weeks, all the groups were thawed at 25 °C (1 min) and 37 °C (4 min). The groups with sucrose or L-proline were diluted in a solution with 0.2 M sucrose or 100 mM L-proline and maintained at 4 °C for 15 min [28]. Subsequently, the cells were centrifuged for 10 min at 600 × g and resuspended in DMEM. After the extracellular cryoprotectant remotion, all the groups were kept for 15 min at 25 °C followed by another centrifugation. Finally, 10 µL cell suspension was evaluated for membrane integrity, and the remaining cells were cultured until 70% confluence and subject to the other analyses described below.

2.6. Evaluation of cell morphology

The cells under an inverted microscope (Leipzig IMx 400, PhoenixOptics, Germany) were evaluated every 48 h for morphology characteristics, such as cytoplasmic projections, nucleus, and the presence of granules or vacuoles [1]. Additionally, medium color changes, the presence of fungal or bacterial contamination, and time until the subconfluence were observed.

2.7. Evaluation of cell membrane integrity, proliferative activity, and metabolism

Cell membrane integrity was evaluated by the cell's permeability to 0.4% trypan blue (in phosphate-buffered saline, PBS) by mixing 1:1 of cell suspension and trypan blue [22]. The viability was evaluated immediately after thawing and after one week of *in vitro* cell culture. Cell numbers were conducted using a Neubauer chamber. Dead cells were permeable to the stain and identified as blue, and viable cells with intact membranes were identified by the absence of color. The viability was determined by cells with intact membrane/total cell counted $\times 100$.

The proliferative capacity was measured by population double time (PDT) in hours [18]. A 7.0×10^4 cells/mL concentration was initially distributed into 7 wells of 24-well dishes. Each dish was counted in intervals of 24 h for up to 168 h. Daily, cells were trypsinized (0.25% trypsin-EDTA solution) and quantified using a Neubauer chamber, and concentrations were used to elaborate the growth curve. The PDT was measured according to the formula: $PDT = T \ln 2 / \ln (X_e / X_b)$, where T is the incubation time, X_b is the number of cells at the beginning of the incubation period, X_e is the number of cells at the end of the incubation period, and $\ln$ is the natural logarithm.

1633

1634 The metabolism was determined by the cell's ability to metabolize 3-(4,5-dimethyl-2-thiazolyl)-
1635 2,5-diphenyl-2H-tetrazolium bromide (MTT assay) in formazan crystals [28]. Initially, a $5.0 \times$
1636 10^5 cell/mL concentration was cultured for five days in the same culture conditions, and then
1637 incubated in MTT solution (5 mg/mL in DMEM) for 3 h at 38.5 °C and 6.5% CO₂. After the
1638 metabolization, formazan crystals were diluted with 1.0 mL Me₂SO, and the absorbance was
1639 measured at 595 nm (Shimadzu® UVmini-1240, Kyoto, Japan).

1640

1641 *2.8. Evaluation of apoptosis classification*

1642 The apoptotic classification was measured by fluorescence, where uniform green, non-uniform
1643 green, orange, and red were considered viable, initial apoptosis, later apoptosis, and necrotic
1644 cells, respectively [29]. Briefly, 50 µL cell suspension was exposed to a 2.0 µg/mL acridine
1645 orange solution and 10 µg/mL ethidium bromide for 4 min and images were captured using a
1646 fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at 40× magnification. For each
1647 group, 300 cells were counted and classified using the cell counter of Image J software (National
1648 Institutes of Health, NIH, USA).

1649

1650 *2.9. Evaluation of reactive oxygen species (ROS) levels and mitochondrial membrane potential* 1651 *($\Delta\Psi_m$)*

1652 ROS levels and $\Delta\Psi_m$ were evaluated using 2',7'-dichlorodihydrofluorescein diacetate
1653 (H₂DCFDA) and MitoTracker Red (CMXRos) probes, respectively. For each probe, cells and

probes were mixed (1:1) and incubated for 30 min at 38.5 °C and 6.5% CO₂ [18]. Subsequently, stained cells were photographed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at 40× magnification. Photomicrographs were evaluated for fluorescence intensity (pixels) using Image J software. Each cell area ~150 μm² was used to measure the fluorescence intensity. The background fluorescence was measured per cell. The final value was the result of the: cell fluorescence = initial cell fluorescence - background fluorescence.

2.10. Statistical analysis

All the data were expressed as mean ± standard error (one animal/ repetition), and analysis was performed using GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA). The Shapiro-Wilk and Levene tests verified the normality and homoscedasticity, respectively. Data was not normally distributed hence arcsine transformed and analyzed by ANOVA followed by the Tukey test. Significance was set at P<0.05.

3. Results

3.1. Evaluation of cell morphology

Before cryopreservation, cells showed fibroblast morphology with a fusiform shape, central and oval nucleus, cytoplasmatic projection, and translucent cytoplasm (Fig. 1A). After cryopreservation (Fig. 1B-G), all the solutions conserved the cell morphology, and the time for 70% confluence was approximately 15 days for all the groups.

3.3. Evaluation of cell membrane integrity, proliferative activity, and metabolism

Immediately after thawing, 2.5% Me₂SO with L-proline ($80.0\% \pm 1.8$) and 10% Me₂SO ($79.4\% \pm 6.1$) promoted higher rates of membrane integrity when compared to the other cryopreserved groups with values between 61% and 69% (Fig. 2A). However, regardless of the cryoprotective solution, the membrane integrity after *in vitro* culture were kept over than 90%.

Moreover, no difference was observed between non-cryopreserved and cryopreserved cells for PDT (Fig. 2B). All PDT showed values ranging from 30 h to 70 h ($P > 0.05$). Moreover, 10% Me₂SO and 10% Me₂SO-sucrose had a slightly higher PDT of 70 h when compared to 2.5% Me₂SO-sucrose and 2.5% Me₂SO-L-proline around 30 h. Additionally, all the groups showed growth curve similarities, with defined latency, exponential, stationary, and plateau phases (Fig. 2C).

Regardless of the cryoprotective solution, cryopreservation did not affect metabolism ($P > 0.05$), ranging from 60% to 100% (Fig. 2D). In particular, the group 2.5% Me₂SO-sucrose ($100.0\% \pm 1.8$) had slightly higher metabolism than the groups 10% Me₂SO ($63.8\% \pm 7.9$), 10% Me₂SO-sucrose ($63.3\% \pm 8.7$) and 10% Me₂SO-L-proline ($60.2\% \pm 10.3$).

3.4. Evaluation of apoptosis classification

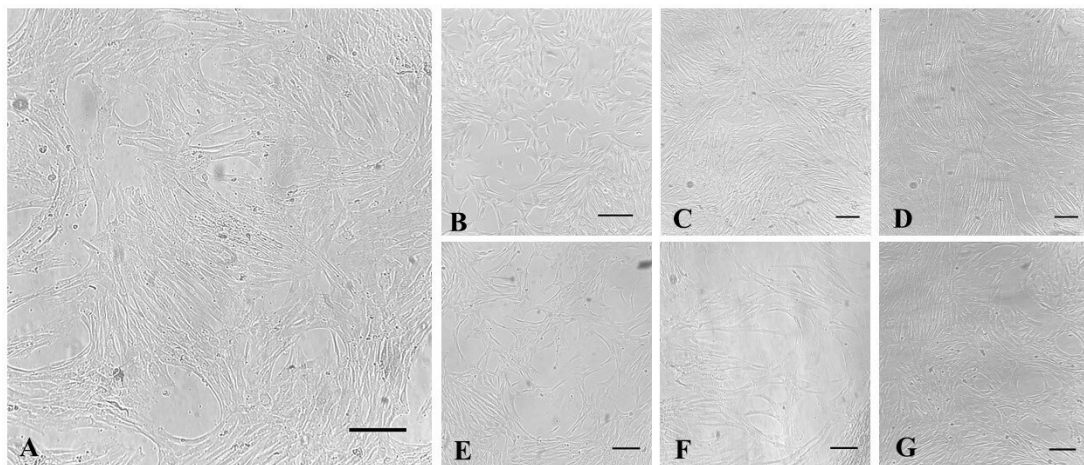
The different levels of apoptotic cells were quantified for all groups (Fig. 3A-G). The 10% Me₂SO-L-proline solution decreased ($P < 0.05$) the viable cells ($81.3\% \pm 9.6$) and increased the

number of late apoptotic cells ($8.3\% \pm 5.5$, Fig. 3H) compared to non-cryopreserved cells. All the other solutions maintained the viable cell number similar ($P > 0.05$) to non-cryopreserved cells ($98.7\% \pm 0.4$, Fig. 3H). Additionally, these solutions showed a lower number of initial apoptotic ($\sim 2\%$), late apoptotic ($\sim 1\%$), and necrotic cells ($\sim 1\%$).

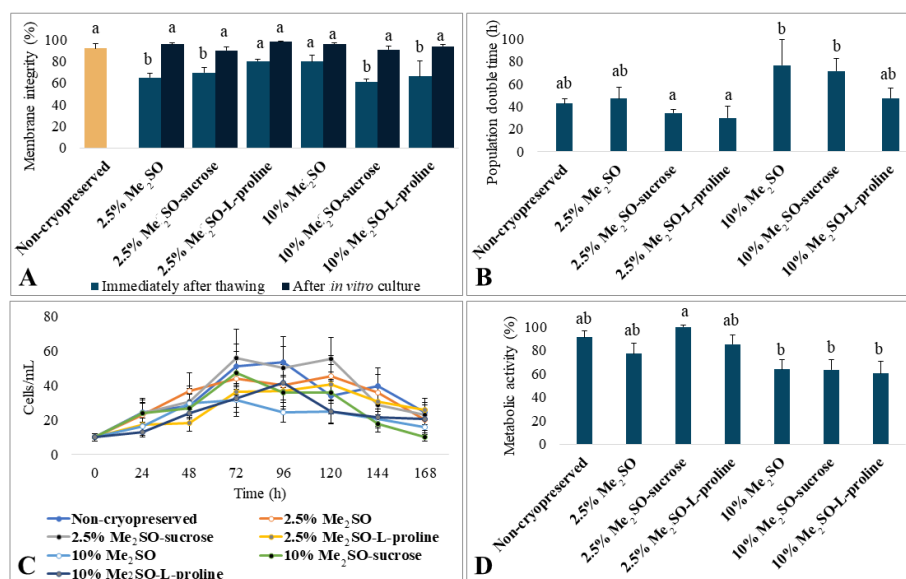
3.5. ROS levels and $\Delta\Psi_m$

The solution with 10% Me₂SO did not affect ($P > 0.05$) the ROS levels when compared to non-cryopreserved cells (Fig. 4A-B). Moreover, the other solutions decreased or increased ($P < 0.05$) ROS levels when compared to non-cryopreserved cells.

All the groups increased the mitochondrial membrane potential (Fig. 4C) compared to non-cryopreserved cells (1.00 ± 0.62 AFU, Fig. 4D). The 2.5% Me₂SO-sucrose was with the higher $\Delta\Psi_m$ (4.92 ± 1.23 AFU) when compared to the other solutions ranging from 3.24 AFU to 3.91 AFU.



1711 **Figure 1.** Morphology evaluation of northern tiger cat fibroblasts subjected to cryopreservation
 1712 in different solutions. **(A)** Non-cryopreserved cells and cells cryopreserved using **(B)** 2.5%
 1713 Me₂SO; **(C)** 10% Me₂SO; **(D)** 2.5% Me₂SO with sucrose; **(E)** 2.5% Me₂SO with L-proline; **(F)**
 1714 10% Me₂SO with sucrose; **(G)** 10% Me₂SO with L-proline; Scale bars: 50 μ m. Magnification:
 1715 20 $\times$.
 1716



1717

1718 **Figure 2.** Membrane integrity and proliferative and metabolic activities of northern tiger cat
 1719 fibroblasts after cryopreservation using different cryoprotectant solutions. **(A)** Cell membrane
 1720 integrity; **(B)** Population double time (PDT); **(C)** Growth curve after 7 days of cell culture; **(D)**
 1721 Metabolic activity. ^{a,b}: P < 0.05.

1722

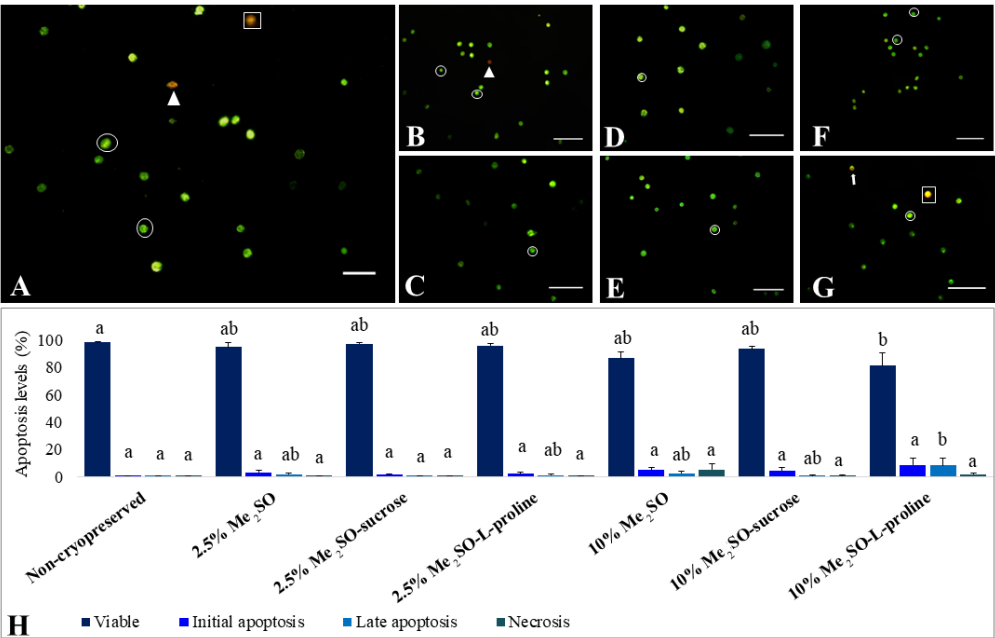


Figure 3. Measurement of apoptotic levels of northern tiger cat fibroblasts after cryopreservation using different cryoprotectant solutions. Representative image of non-cryopreserved cells (A) and cells cryopreserved with 2.5% Me₂SO (B); 10% Me₂SO (C); 2.5% Me₂SO with sucrose (D); 10% Me₂SO with sucrose (E); 2.5% Me₂SO with L-proline (F); 10% Me₂SO with L-proline (G); Circle: Viable cell; Square: Initial apoptosis; Triangle: Late apoptosis; Arrow: Necrosis. (E) Quantification of apoptotic levels; Scale bars: 50 μ m. Magnification: 40 $\times$.

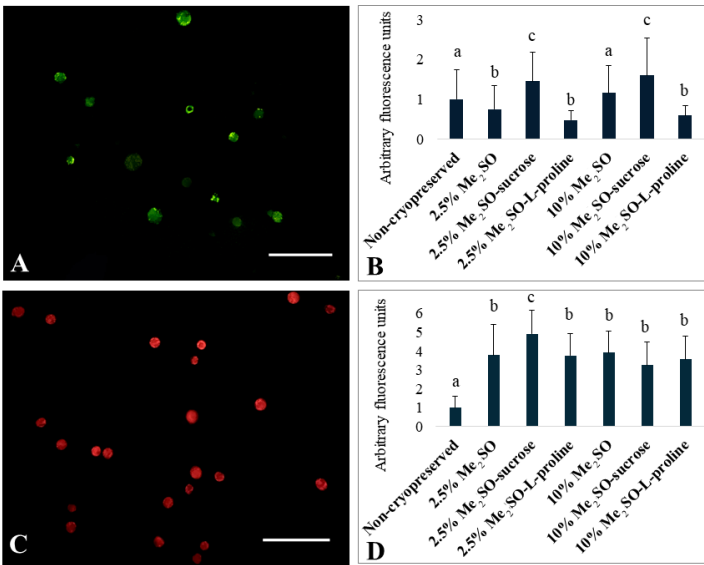


Figure 4. Oxidative stress measured by the ROS levels and mitochondrial membrane potential ($\Delta\Psi_m$) of northern tiger cat fibroblasts after cryopreservation using different cryoprotectant solutions. **(A)** Non-cryopreserved cells marked with MitoTracker Red; **(B)** Mitochondrial membrane potential measurement; **(C)** Non-cryopreserved cells marked with H₂DCFDA; **(D)** Quantification of ROS levels. Scale bars: 50 μ m. Magnification: 40 $\times$. ^{a,b,c} P < 0.05.

4. Discussion

In this study, we observed no synergistic effect of sucrose and L-proline with reducing the concentration of Me₂SO on the cryosurvival of northern tiger cat fibroblasts. In wild felids, adding extracellular cryoprotectants, such as sucrose, can improve cells' metabolic activity and PDT [17]; however, this effect was not observed in northern tiger cat somatic cells. Reducing cryoprotectant concentration has been recognized as a strategy to decrease the toxicity during cell cryopreservation. Arantes et al. [1] reported >80% of viable somatic cells after cryopreservation with 2.5% Me₂SO of northern tiger cats. In our study, the viability of cells cryopreserved with 2.5% Me₂SO decreased immediately after thawing. Klbbik et al. [13] described the effect of a lower concentration of Me₂SO (~2%) causing a more significant ice crystal formation and decreasing cell viability by exposure to higher pressure. The influence of Me₂SO concentration is clear, due to the 10% Me₂SO likely preserved higher viability immediately after thawing for northern tiger cat somatic cells.

Moreover, the presence of sucrose did not improve the viability immediately after thawing. Similar results were observed for adult skin fibroblasts of Iberian lynx [17]. Fortunately, the addition of 100 mM L-proline to 2.5% Me₂SO improved this somatic cell viability. Bailey et al. [3] showed that 100 mM L-proline inhibited ice crystal development, increasing the viability from 25% to 60% in human lung carcinoma cells. This ability is especially dependent on

extracellular cryoprotectant characteristic as hydration ability, cryoprotectant concentration, and the type and species [5]. -

Interestingly, all the groups promoted the conservation of metabolic activity. Whaley et al. [31] described the function of Me₂SO as decreasing the membrane thickness, resulting in a permeability increase and promoting less intracellular ice formation. This mechanism could improve cell cryosurvival even without combining cryoprotectants. Moreover, the solution with 2.5% or 10% Me₂SO alone or with sucrose protected the metabolic activity of puma fibroblasts [28].

Additionally, great results were reported on cell proliferation of northern tiger cat cells in our study. Chaytor et al. [5] described the Me₂SO action as inhibiting ice recrystallization at lower concentrations (2.5%), especially when combined saccharides. Moreover, the cryopreservation of Jurkat cells with 5% Me₂SO and 100 mM L-proline maintained cell proliferation, similar to our study with both Me₂SO concentrations [21].

Furthermore, the antioxidant effect of L-proline in our study acted decreasing ROS levels and increased $\Delta\Psi_m$ in all solutions with Me₂SO. Similarly, Len et al. [15] described that L-proline during cryopreservation act reducing ROS levels by connecting with free radicals and increasing the $\Delta\Psi_m$. Inversely, sucrose increased the ROS levels. These levels can be caused by Me₂SO promoting the permeability of the cell membrane, an increase of sucrose can pass through the

membrane, resulting in cell stress [9, 23]. Unlike the solution with extracellular cryoprotectant or only 2.5% Me₂SO, the solution with 10% Me₂SO maintained the ROS levels. These results occur due to the Me₂SO intracellular protective action, probably decreasing the ice growth and recrystallization during thawing [4, 11].

Unfortunately, L-proline resulted in a decrease in viable cells by apoptosis levels assay. L-proline has been used as a substitute for Me₂SO to avoid the toxicity effect and promote conservation with a substance with similar osmolality [6, 19]. In our study, the increase of Me₂SO concentration in the presence of L-proline could have changed the osmotic equilibrium during the dehydration of slow freezing and affected the protein stability, resulting in cell death [32].

Finally, adding L-proline or sucrose in solution with 2.5% or 10% Me₂SO did not improve the cell parameters compared to control groups with only Me₂SO. Arantes et al. [1] described the success of northern tiger cat somatic cell cryopreservation with a solution without Me₂SO or extracellular cryoprotectant; northern tiger cat somatic cells probably presented better results with simpler solutions. Awan et al. [2] showed that Me₂SO can have a variable effect on different cell types, concentrations, and mechanisms, resulting in better results alone than when combined with other cryoprotectants.

5. Conclusions

Even with several studies showing the efficiency of sucrose or L-proline on somatic cell

cryopreservation, adding these substances did not improve the cryopreservation of northern tiger cat fibroblasts. Moreover, reducing the concentration of Me₂SO to 2.5% had no impact on the efficiency of cryopreservation. Therefore, 10% Me₂SO was determined to be most effective for cryopreserving northern tiger cat fibroblasts.

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Conflict of interest

The authors declare no conflict of interest.

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1925	CAPÍTULO 4- THE ROLE OF SOMATIC CELL SYNCHRONIZATION IN NUCLEAR
1926	TRANSFER AND INDUCED PLURIPOTENT STEM CELLS FOR WILD FELIDS
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1954 **The role of somatic cell synchronization in nuclear transfer and induced pluripotent stem**
 1955 **cells for wild felids**

1956

1957 João V.S. Viana & Alexsandra F. Pereira*

1958

1959 Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoró, RN,
 1960 Brazil

1961

1962 ***Correspondence:** Alexsandra F. Pereira, PhD, Laboratory of Animal Biotechnology, Federal
 1963 Rural University of Semiarid, Av. Francisco Mota, 572, Costa e Silva, Mossoró, RN, Brazil,
 1964 59625900, E-mail: alexsandra.pereira@ufersa.edu.br.

1965

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1970 **ABSTRACT**

1971 Human interference reduces wild felid populations. Somatic cell nuclear transfer and the use of
 1972 induced pluripotent stem cells are potential conservation strategies. To improve the efficiency of
 1973 these strategies, it is essential to establish adequate protocols for the synchronization of cells in
 1974 the G0/G1 phase of the cell cycle. Cell cycle synchronization can arrest cell cycle progression by
 1975 inhibiting factors involved in cell duplication. However, this step varies among wild felids and
 1976 has not been successful in some species. In addition, the effect of this step on cell applications
 1977 remains unclear. Therefore, this review highlights the primary differences among wild felids that

can cause this variability, the most promising results, and the methods used. Finally, the importance of cell cycle synchronization in biotechnologies involving the nuclear reprogramming of somatic cells in wild felid conservation is highlighted.

KEYWORDS: wildlife, conservation strategies, fibroblast, nuclear reprogramming, cloning

1. INTRODUCTION

Human interference disrupts the natural balance and decreases mammalian populations. This is most evident in the Felidae family, with 47% of species in this family categorized as in threat of extinction (Fernández-Sepúlveda, & Martín, 2022). Population reductions of these species have been accompanied by ecosystem imbalances, especially in nutrient distribution and genetic diversity (Karandikar et al., 2022). Therefore, advances in conservation methods are essential.

In situ and *ex situ* conservation strategies have been developed and are correlated with increased biodiversity (Luczinski et al., 2023). Focusing on *ex situ* conservation, several countries have studied new possibilities for implementing associative systems between zoos and storing biological materials for use in assisted reproductive technologies (ARTs; Kochan et al., 2019; Swanson, 2023). Embryos, gametes, and gonadal tissues are the first choices for animal conservation, and advances in cloning via somatic cell nuclear transfer (SCNT; Veraguas, Gallegos, Castro, & Rodríguez-Alvarez, 2017) and the production of induced pluripotent stem cells (iPSCs; Verma, Liu, Holland, Temple-Smith, Williamson, & Verma, 2013) have promoted the establishment of fibroblast cryobanks for wild felids.

In general, using fibroblasts in reprogramming techniques requires synchronization of the cell cycle in G₀/G₁. The cell cycle is defined by a series of events regulated by a group of cyclin-

dependent protein kinases (CDKs) that promote the correct progression to the G₁, S, G₂, and M phases. The quiescent state of a cell is promoted during cell cycle synchronization (Wang, 2021). Cell cycle arrest correlated with increased homogeneity and CDK regulation, resulting in improved vector infection for iPSC generation (Zhao et al., 2016). Similarly, cell cycle arrest is necessary for SCNT to oocytes in metaphase II and somatic cells, allowing for the correct replication of genetic material (Thongphakdee, Sukparangsi, Comizzoli, & Chatdarong, 2020).

Several cell cycle synchronization methodologies have been applied, based on chemical agents (Hashem, Bhandari, Kang, & Lee, 2007), contact inhibition (CI), or serum starvation (SS) (Młodawska et al., 2022). Different methods promote the inhibition of cell proliferation via the suppression of CDK or other mechanisms. Promising results have been observed in wild felids when using chemical agents with a G₀/G₁ cycle synchronization efficiency of 80% and CI and SS values of approximately 85–95% in some species (Wittayarat, Thongphakdee, Saikhun, Chatdarong, Otoi, & Techakumphu, 2013; Yelisetti et al., 2016). However, the results have been highly variable, and for some species, all methodologies have been ineffective, with percentages less than 75% (Hashem et al., 2007).

Although protocols for cell cycle synchronization in G₀/G₁ have been proposed, the variability observed between felid species in the percentage of G₀/G₁ cells, an increase in cells in sub-G₀/G₁, or a reduction in cell viability (Młodawska et al., 2022), has generated interest among different groups to better understand the reasons for these responses. This review describes the primary findings, challenges, and perspectives of G₀/G₁ synchronization methodologies used in SCNT and iPSC technologies in wild felids.

2. CHALLENGES OF G₀/G₁ CELL SYNCHRONIZATION

Obtaining a fibroblast line, which involves cell isolation and storage, is the first step toward synchronizing the cell cycle in G₀/G₁ (Bolton et al., 2022). In this step, three parameters are essential: (i) choosing the least-invasive region of the animal for sampling, (ii) transport time, and (iii) aseptic conditions. These are critical challenges in recovering viable cells from animals with natural resources (Sano et al., 2022).

Aseptic conditions have been a challenge when establishing cell lines for species, including the jaguar (*Panthera onca*), but this can be addressed by better preparation of biopsy specimens under aseptic conditions and medium color control, in which the culture dish should be discarded if the medium changes to yellow (Mestre–Citrinovitz et al., 2016). Confirmation of the absence of contamination is important for cell applications, and this has been analyzed in several species, such as the Siberian tiger (*Panthera tigris altaica*) (Liu et al., 2010) and Bengal tiger (*Panthera tigris tigris*) (Guan et al., 2010). Moreover, transport and storage time influences the recovery of fishing cats (*Prionailurus viverrinus*) from natural resources when the tissue is stored for 1–3 days at 4°C (Sukparangsi et al., 2022).

Moreover, *in vitro* culture conditions can affect cell quality through the damage caused by oxidative stress and the number of passages (Place, Domannb, & Case 2017). In fishing cats, the removal of GlutaMAXTM reduces cell metabolism, with cells affected at the fifth passage (Sukparangsi et al., 2022) because of their lower density. In contrast, puma (*Puma concolor*) medium without supplementation promotes cell proliferation until the 10th passage (Lira et al., 2022). Variability in the cell culture media of other wild felids is shown in Table 1.

2050

2051 In this sense, specific characterization of the cell line may be the key to successful cycle
2052 synchronization, because knowledge of each species' doubling time and cell dynamics allows the
2053 appropriate use of cycle synchronization protocols. Arantes, Tonelli, Martins, & Báo (2021)
2054 described cell isolation from three wild felids: jaguar, northern tiger cat (*Leopardus tigrinus*), and
2055 pampas cat (*Leopardus colocolo*). The three species showed differences in their cellular
2056 ultrastructure and growth curves. Several other studies have reported variations in these
2057 characteristics among wild felids (Table 1).

2058 3. METHODOLOGIES FOR G₀/G₁ CELL SYNCHRONIZATION

2059 Cell cycle synchronization has been described as a method that can block proliferation under cell
2060 culture conditions or inhibit specific molecules (for example, CDK), and this block should be
2061 reversible and non-toxic (Banfalvi, 2017). Cells normally have checkpoints in the cell cycle;
2062 however, synchronized cells are unable to enter the cell cycle because of the absence of growth
2063 factors and the inhibition of CDK (Davis et al., 2001). An example of cell cycle synchronization
2064 conditions is shown in Figure 1.

2065

2066 Cells typically present an asynchronous cycle during *in vitro* culture, thus highlighting the
2067 importance of cell cycle synchronization methodologies for SCNT studies (Cooper, 2003) and the
2068 uniformity of iPSC (Leal, Herreno-Pachón, Benincore-Flórez, Karunathilaka, & Tomatsu, 2024).
2069 These methods can be used in the presence of chemical agents that act through molecular
2070 inhibition or in the absence of chemical components through nutritional deprivation (SS) or CI
2071 (Eastman, & Guo, 2020).

2072

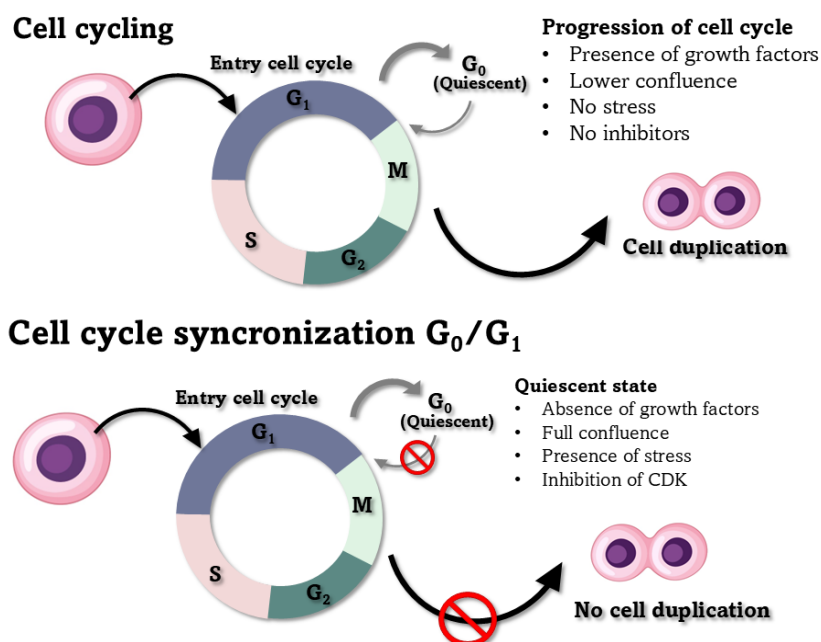


Figure 1. Representative scheme of cells cycling and cells subjected to synchronization in G₀/G₁.

In wild felids, roscovitine, which inhibits CDK, is most studied at concentrations from 7.5 to 30 μ M, with incubation times from 12 to 24 h (Wittayarat et al., 2013; Rodrigues et al., 2023). Furthermore, Yelisetti et al. (2016) showed interesting results with the inhibition of histone deacetylase by sodium butyrate at concentrations of approximately 0.5 to 3.0 mM for 24 or 48 h. Hashem et al. (2007) highlighted the possibility of using a protease inhibitor (6-dimethylaminopurine [6-DMAP]), cycloheximide, cytochalasin B, and antioxidants (β -mercaptoethanol, cysteine, and glutathione) for 4 h. With significant variability, it is essential to determine the ideal chemistry and incubation time for each species.

2086 **TABLE 1.** Difference between the culture conditions and cell proliferation of fibroblasts derived from wild felids.

Species	Culture medium	Passage number	PDT (in h)	Growth curve	Authors
<i>Panthera tigris altaica</i>	DMEM supplemented with 10% FBS, and 1% non-essential amino acid	8 th passage	17.2	Exponential phase until 144 h and stationary phase in 168 h	Song et al. (2007)
<i>Lynx pardinus</i>	DMEM supplemented with 1% non-essential amino acids, 0.1 mM 2-mercaptoethano, 1 mM sodium pyruvate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin	1 st passage	48 at 240	Growth curve was not analyzed	Léon-Quinto et al. (2011)
<i>Panthera leo persica</i>			26.7	Exponential phase until 168 h and stationary phase in 168 h	

<i>Panthera tigris tigris</i>		27.2	Exponential until 144 h and stationary phrase after 192 h	
<i>Panthera pardus fusca</i>	DMEM supplemented with 20% FBS, and 2% antibiotics	34.7	The exponential phase until 144 h and stationary phrase after 168 h	Yelisetti et al. (2016)
<i>Leopardus tigrinus</i>		24	Exponential phase after 120 h	
<i>Leopardus colocolo</i>	DMEM supplemented with 10% FBS, with 2% penicillin G and streptomycin	24	Exponential phase after 192 h	Arantes et al. (2021)
<i>Panthera onca</i>		24	Exponential phase until 70 h and stationary phase after 80 h	

<i>Panthera onca</i>	DMEM supplemented with 10% FBS, and 2% antibiotic/antimycotic solution	1 st , 3 rd , and 10 th passage	22.8 at 26	The exponential phase until 72 h, stationary phase until 120 h and cell decrease after 144 h	Silva et al. (2021)
<i>Puma concolor</i>	DMEM supplemented with 10% FBS, and 2% antibiotic/antimycotic solution	1 st , 3 rd , and 10 th passage	31	Exponential phase until 96 h, stationary phase until 144 h and decrease on 168 h	Lira et al. (2022)

2087 DMEM: Dulbecco's Modified Eagles Medium; FBS: fetal bovine serum.

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Without a chemical agent, nutrient deprivation inhibits the growth stimulus and promotes a quiescent state in G₀/G₁ (Davis et al., 2001). Several studies have shown effective results with SS by changing the concentration of fetal bovine serum (FBS) in the medium from 10% to 0.5% for 24–120 h (Yelisetti et al., 2016; Veraguas et al., 2017). The quiescent state can also be induced by stress caused by cell contact at 100% confluence and nutrient deprivation for 24 to 120 h (Wittayarat et al., 2013; Rodrigues et al., 2023). The combination of the two methodologies has shown effectiveness, with values of approximately 90% G₀/G₁ cells in *Puma yagouaroundi*, after incubation times of 24 to 120 h (Młodawska et al., 2022).

3.1. Chemical inhibitors

Chemical inhibitors have been developed based on the molecule responsible for the regulation of the cell cycle, which is the most conserved molecule in the CDK group, where CDK1 plays an important role in cell cycle progression in combination with other CDKs (Krasinska, Cot, & Fisher, 2008). CDK inhibitors include roscovitine, which specifically inhibits the binding of CDK 1, 2, 5, and 7 to the ATP regions of molecules, with lower concentrations correlated with less cytotoxic effects (Wu et al., 2008).

The effects of roscovitine have been described in wild felids (Table 2), with efficiency observed only in the African wild cat (*Felis silvestris libica*) and Asian golden cat (*Pardofelis temminckii*). This has led to the development of cloned blastocysts of African wild cats (Gómez et al., 2003). In general, the incubation time was 12–24 h. Therefore, increasing concentration may be a viable strategy. However, Wittayarat et al. (2013) observed that 30 µM caused the most apoptotic cells in the Asian golden cat and leopard (*Panthera pardus*). In summary, new protocols should be developed based on the results observed in other wild felids with changes in the incubation time, between 12 and 24 h, and the concentration around 7.5, 15, and 30 µM.

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2118 Alternative methods, such as sodium butyrate, a histone deacetylase (HDAC) inhibitor, have been
 2119 tested in wild felids and have shown a significant increase in the percentage of synchronized cells
 2120 to approximately 70% of cells in G₀/G₁ (Table 2). Yelisetti et al. (2016) showed no toxic effect of
 2121 1.0 mM sodium butyrate with 48 h of incubation for the Asiatic lion (*Panthera leo persia*), Indian
 2122 tiger (*Panthera tigris tigris*), and Indian leopard (*Panthera pardus fusca*) in interspecies nuclear
 2123 transfer (iSCNT), with approximately 5% blastocysts.

2124

2125 Hashem et al. (2007) tested the ability of antioxidants to increase oxidative stress and cell cycle
 2126 interference, including a protein kinase inhibitor (6-DMAP), a protein synthesis inhibitor
 2127 (cycloheximide), and a cell division inhibitor (cytochalasin B). Alternative chemical tests are
 2128 interestingly used strategies that use different methodologies to block cell-cycle synchronization.
 2129 They will help us understand the best methodology for improving cell synchronization.
 2130 Therefore, the chemical mechanism is essential to avoid DNA damage, the pro-apoptotic gene
 2131 *BAX*, decreased viability, and cell detachment, as shown by methods, such as CI and SS
 2132 (Veraguas et al., 2017).

2133 **3.2. Serum starvation (SS) and contact inhibition (CI)**

2134 The SS method at 0.5% FBS for 120 h was the first used to clone sheep (Wilmut, Schnieke,
 2135 McWhir, Kind, & Campbell, 1997). This method has some benefits, such as no special reagent
 2136 requirements; the low cost of the change, as it is only a change in culture medium conditions; and
 2137 its effectiveness with several cell types (Jackman, & O'Connor, 2001). The SS and CI methods
 2138 mediate cell cycle arrest by inducing stress. Both methods increase the concentration of p27, an
 2139 inhibitor of CDK, and arrest the cell cycle (Davis et al., 2001; Pack, Daigh, & Meyer, 2019).

2140 **TABLE 2.** Studies using chemical agents in wild felid cells for G₀/G₁ cycle synchronization.

Chemical	Species	Better Concentration	Time (in h)	Sub-G ₀ /G ₁ cells (in %)	G ₀ /G ₁ cells (in %)	Authors
Roscovitine	<i>Felis silvestris libica</i>	15 µM	24	-	~56	Gómez et al. (2003)
	<i>Pardofelis temminckii</i>	7.5 or 15 µM		~2	~93	
	<i>Pardofelis marmorata</i>	7.5; 15 or 30 µM (No effect for species)	24	~1	~80	Wittayarat et al. (2013)
	<i>Panthera pardus</i>	7.5; 15 or 30 µM (No effect for species)		~1	~80	
	<i>Puma concolor</i>	15 µM (No effect for species)	12 or 24	~2	~80	Rodrigues et al. (2023)

Sodium butyrate	<i>Panthera leo persica</i>	0.5 mM	48	~0.5	~76	Yelisetti et al. (2016)
	<i>Panthera tigris</i>	0.5 mM	24	~0.3	~65	
	<i>Panthera pardus fusca</i>	0.5 mM	48	~0.5	~73	
β-mercaptoethanol		10 μM			~60	Hashem et al. (2007)
Cysteine		2 mM			~55	
Glutathione	<i>Panthera tigris</i>	2 mM			~73	
6-DMAP	<i>altaica</i>	2 mM	4	-	~60	
Cycloheximide		7.5 μg/mL			~75	
Cytochalasin B		7.5 μg/mL			~61	

- : Not analyzed for this study.

The use of SS and CI has been effective for most wild felid species (Table 3), with more significant variance in the times from 1 (24 h) to 5 days (120 h). In particular, SS results in close to 100% of synchronized cells, and thus, it may be considered more effective than chemical methods, which have values closer to 70–80% of G₀/G₁ cells. Hashem et al. (2007) compared the SS and CI of the chemicals tested (Table 2) and reported higher of G₀/G₁ for CI values when compared to some chemicals. Embryo production via SCNT, SS, and CI has been effective in African wild cats (~21–28% of embryos, Gómez et al., 2003), Asiatic lions, Indian tigers, and Indian leopards (~3–4% of embryos, Yelisetti et al., 2016).

Despite these promising results, alternative methods of cell synchronization are essential because the SS and CI methods can increase the number of apoptotic cells, and stress causes an increase in autophagy, senescence, and DNA fragmentation (Młodawska et al., 2022). Veraguas et al. (2017) described the effect of CI and SS on the relationship between *BAX* and *BCL2* genes, reporting an increase in the expression levels of these genes after 3 and 5 days of treatment. This was especially true for CI, which was considered to be ineffective because of its effect on viability.

4. CELL SYNCHRONIZATION AS A STEP FOR SOMATIC CELL APPLICATIONS

The applicability of SCNTs and iPSCs for somatic cells has been widely described. Several steps are performed during these procedures (Figure 2). The first steps for SCNT and iPSCs have been studied using cell culture and cryopreservation in 27 felid species. The next step is cell cycle synchronization, which has been performed for 18 species. Similarly, SCNT has been studied in only 14 species and only two species have shown birth after SCNT. These results are because of the complexity and multiple factors involved in iPSC and SCNT studies. Postmortem somatic

tissue cryopreservation can also be used for cell recovery and applications in SCNT (Moulavi et al., 2017). However, this step has been established for only five species. Additionally, none of the studies on the use of iPSCs in wild felids have established a correlation between cell cycle synchronization and improvements in the results of iPSC use.



Figure 2. Studies with somatic tissue and cells in wild felids.

4.1. Correlation between SCNT efficiency and the cell cycle synchronization

The first step in the development of cloned embryos is to determine the quality of the nuclear donor and recipient oocytes. During this process, the nuclear donor should stay synchronized with the recipient for the correct nuclear reprogramming of cells (Swegen, Appeltant, & Williams, 2023).

2179 **TABLE 3.** Effect of SS and CI on G₀/G₁ cells of wild felids.

Species	Method of synchronization	Time of treatment (in h)	Sub G ₀ /G ₁ cells (in %)	G ₀ /G ₁ cells (in %)	Authors
<i>Felis silvestris libica</i>	SS	120	-	~97	Gómez et al., 2003
	CI	120	-	~88	
<i>Panthera Tigris</i>	SS	120	-	~66	Hashem et al. (2007)
<i>Altaica</i>	CI	120	-	~68	
<i>Pardofelis temminckii</i>	SS	24, 48, 72, or 120	~2,5	~95	Wittayarat et al. (2013)
	CI	120	~3	~95	
<i>Pardofelis</i>	SS	48 or 72	~2	~95	
	CI	120	~2	~83	
<i>marmorata</i>					
(No effect for species)					

<i>Panthera pardus</i>	SS	48, 72, or 120	~2	~95	
	CI	120	~1	~90	
<i>Panthera leo persica</i>	SS	24	~1,6	~85	
	CI	24	~1.2	~91	
<i>Panthera tigris tigris</i>	SS	24	~1	~90	Yelisetti et al. (2016)
	CI	24	~2	~92	
<i>Panthera pardus</i>	SS	24	~1	~88	
<i>fusca</i>	CI	24	~2	~90	
<i>Leopardus guigna</i>	SS	120	~2	~83	Veraguas et al. (2017)
	CI	24, 72, and 120	~2	~72	
		(not effective)			
<i>Puma yagouaroundi</i>	SS	120	-	~97	Młodawska et al.

<i>Felis manul</i>	CI	72	-	~94	(2022)
	SS	24	-	~95	
	CI	24	-	~93	
<i>Puma concolor</i>	SS	96	~3	~86	Rodrigues et al.
	CI	24	~6	~85	(2023)

- : Not analyzed for this study.

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The quiescent state promoted during the G₀/G₁ phase makes it possible to correct DNA replication and the accessibility of chromatin in the oocytes, which, with high quantities of maturation-promoting factors when activated, promotes DNA exposition at the replication factors of oocytes and reprogramming (Gouveia, Huyser, Egli, & Pepper, 2020).

iSCNT studies involving SS and CI, with successful results indicating the possibility of blastocyst development and animal birth (Gómez et al., 2004). When comparing chemical agents with SS or CI for SCNT, only sodium butyrate demonstrated higher effectiveness than CI in lion blastocyst production (Yelisetti et al., 2016). However, the effect of roscovitine on wild felid blastocysts has not been well documented, and roscovitine does not yield better results than CI or SS.

In summary, even in Siberian tigers, where all methods have resulted in less than 70% of synchronized cells, SS and CI were the methods of choice for blastocyst production (Hashem et al., 2007); however, they resulted in the development of only one or two blastocysts. Reviewing the findings of previous studies to determine the best methodology for cell synchronization is essential because SS and CI are not effective for some species and chemical agents are not as effective as SS and CI for some species.

4.2 Can cell cycle synchronization improve the iPSC generation on wild felids?

iPSCs can be reprogrammed using transcription factors such as OCT4, SOX2, KLF4, C-MYC, and NANOG (Verma et al., 2012; 2013). Variability among wild species in the effectiveness of different viral vectors, specific factor combinations, and media for iPSC proliferation *in vitro* has been described (Kumar, Talluri, Selokar, Hyder, & Kues, 2021). iPSCs have been developed for

wild felids with the aim of applying these cells to germ cell development, genetic material restoration, and regenerative medicine (Liu, David, Trawczynski, & Fessler, 2020).

Studies of iPSCs in wild felids have consisted of testing transfection methods with combinations of factors and assessing cell culture media and different coating surfaces. However, these studies have failed to produce fishing cat iPSCs (Sukparangsi et al. 2022). Verma et al. (2012, 2013) obtained iPSCs for the snow leopard (*Panthera uncia*), Bengal tiger (*Panthera tigris*), serval (*Leptailurus serval*), and jaguar and found no silenced factors after 14 passages. Moreover, other studies have focused on the effects of various factors (Verma et al., 2012; 2013), media, and vectors (Sukparangsi et al., 2022). To date, no studies have been conducted on the effects of cell synchronization on iPSC generation.

Moreover, the quiescent state of synchronized cells can improve cell reprogramming by inhibiting CDK, because the addition of CDK decreases the efficiency of cell reprogramming (Hindley & Philpott, 2013). Therefore, studies on cell cycle synchronization during iPSC generation for wild felids are necessary, especially in species for which the insertion of reprogramming factors, such as the fishing cat, is not effective.

5. CONCLUSIONS

Cell cycle synchronization varies among wild felids, with specific methods of choice for each species. Successful cell cycle synchronization requires a high-quality cell line without chromosomal damage, bacterial contamination, or senescence. In general, SS and CI methods have been the most effective for a higher number of species and have been most frequently applied to SCNT compared to chemical methodologies. Moreover, variability in the type of

chemical, type of inhibition, incubation time, and concentration requires further study to increase the efficiency of this method. Finally, cell cycle synchronization is necessary for SCNT; however, the correlation between this step and wild-type iPSC production has not been widely studied. Thus, the establishment of cell cycle synchronization must be studied, and the chemical methods and correlations between this step and the capacity to improve iPSC production should be improved.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Alexsandra F Pereira: <https://orcid.org/0000-0003-2137-854X>

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**CAPÍTULO 5- EXPLORING THE EFFECTS OF ROSCOVITINE, SERUM
STARVATION, AND CONTACT INHIBITION ON G₀/G₁ ARREST IN NORTHERN
TIGER CAT DERMAL FIBROBLASTS**

Artigo experimental: Exploring the effects of roscovitine, serum starvation, and contact
inhibition on G₀/G₁ arrest in northern tiger cat dermal fibroblasts

Periódico de submissão: *In Vitro* Cellular & Developmental Biology - Animal

Fator de impacto: 1,5

Data de Submissão: 06/02/2025

**Exploring the effects of roscovitine, serum starvation, and contact inhibition at G₀/G₁ arrest
in northern tiger cat dermal fibroblasts**

João Vitor da Silva Viana¹, Brenna de Sousa Barbosa¹, Luanna Lorena Vieira Rodrigues¹,
Antonia Beatriz Mendonça Pereira¹, Patrícia Vasconcelos Alves², Herlon Victor Rodrigues
Silva³, Sarah Sant'Anna Maranhão⁴, Carlos Roberto Koscky Paier⁴,
Maria Claudia dos Santos Luciano⁴, Cláudia Pessoa⁴, Alexsandra Fernandes Pereira^{1*}

¹Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoro, RN,
Brazil. ²Sargento Prata Municipal Zoo, Fortaleza, CE, Brazil. ³Laboratory of Carnivores
Reproduction, University of Ceara State, Fortaleza, CE, Brazil. ⁴Laboratory of Experimental
Oncology, Center for Research and Drug Development, Federal University of Ceará, Fortaleza,
CE, Brazil.

*Corresponding author: Alexsandra Fernandes Pereira

Laboratory of Animal Biotechnology

Federal Rural University of Semi-Arid

Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil

Phone: +55 84 3317 8361

Email address: alexsandra.pereira@ufersa.edu.br

2455 **Running head:** Northern tiger cat cells G₀/G₁ arrest

2456 **Abstract**

2457 Nuclear reprogramming studies are important tools in conserving wild felids, with efficacy
2458 depending on efficient G₀/G₁ cell cycle arrest methodologies. This study evaluated different
2459 culture conditions at G₀/G₁ arrest and the quality of northern tiger cat fibroblasts. Cells from four
2460 animals were assigned to groups: 7.5 and 15 µM roscovitine (RSV) for 24 and 48 h, serum
2461 starvation (SS) for 24, 48, 72, and 96 h, and contact inhibition (CI) for 24, 48, and 72 h. Cells
2462 with 50–60% confluence were used as control. The cell quality parameters included morphology,
2463 viability and apoptotic levels were assessed through microscopic analysis, while cell cycle phases
2464 were evaluated using flow cytometry. RSV affected the cell viable percentage and morphology
2465 with the increase of concentration and exposure time. Moreover, RSV did not improve the cells at
2466 G₀/G₁. CI did not significantly affect cell quality or increase the proportion of cells in G₀/G₁
2467 phase. Interestingly, SS for 24 h increased the cells at G₀/G₁. However, SS affected the apoptosis
2468 levels. The SS for 24 h is the most efficient method of G₀/G₁ arrest for northern tiger cat
2469 fibroblasts. However, adjustments are still necessary to optimize cell arrest for northern tiger cat
2470 fibroblasts.

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2472 **Keywords:** wild felids, cell cycle arrest, flow cytometry, serum starvation

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2474 **Introduction**

2475 The population of small wild felids is influenced by habitat quality and interspecies interaction,
2476 making them a good model animal for conservation planning (Cepeda-Duque et al. 2023). This
2477 group includes the northern tiger cat (*Leopardus tigrinus* Schreber, 1775) as a mesopredator,
2478 contributing to ecosystem equilibrium by regulating prey populations (Oliveira et al. 2020).

Regrettably, owing to habitat reduction, human interference, and disease from domestic animals (Anderson et al. 2024), the species has been classified as vulnerable to extinction (Payan and Oliveira, 2016).

Considering this context, conservation strategies to safeguard these species are essential (Resende et al. 2014). An interesting strategy consists of creating fibroblast banks (Praxedes et al. 2018), which, when appropriately established, can be used in association with nuclear reprogramming techniques, such as cloning by somatic cell nuclear transfer (SCNT, Gómez et al. 2008) and production of cells induced to pluripotency (iPSC, Verma et al. 2013). Recently, we have established some **techniques** in creating these banks in northern tiger cats, whether by evaluating the culture time (Viana et al. 2025b) or cryopreservation solutions (Viana et al. 2025a). The next step consists of assessing the culture conditions to increase the percentage of cells at G₀/G₁, aiming at their use in reprogramming studies (Veraguas et al. 2017).

In wild felids, three methodologies have been employed for cell cycle arrest at G₀/G₁. Some of them are roscovitine (RSV) as chemical inhibition (Wittayarat et al. 2012), serum starvation (SS, Yelisetti et al. 2016), and contact inhibition (CI, Młodawska et al. 2022). In general, RSV acts by activating the inhibition of Cyclin-Dependent Kinase (CDK) 1, 2, 5, and 7, inhibiting the ATP binding (Cicenas et al. 2015). However, its efficiency depends on the concentration in a species-specific manner (Rodrigues et al. 2023). In Asian golden cat (*Pardofelis temminckii*) and marbled cat (*Pardofelis marmorata*), RSV at 7.5 and 15 µM after 24 h were efficient for increasing G₀/G₁ in cells (Wittayarat et al. 2012). Moreover, other factors can alter the RSV efficiency, such as population doubling time (Koo et al. 2008) and the period of incubation of chemical inhibitors (Ligasová et al. 2023).

On the other hand, SS and CI are based on changes by a decrease of 0.5% of serum on medium and the highest confluency (>90%). The mechanism of SS and CI is the downregulation of CDK by the privation of necessary extracellular components to keep cell growth (Davis et al. 2001). In wild felids, SS exhibited variable results with increased G₀/G₁ cells of puma (*Puma concolor*) after 96 h but affecting the viability (Rodrigues et al. 2023). In contrast, for marbled cats, the method increased the G₀/G₁ in 24 h without affecting viability (Wittayarat et al. 2012). Similarly, CI showed results dependent on time of exposure with an increase of G₀/G₁ and a decrease of viable cells in puma (Rodrigues et al. 2023), Indian tiger (*Panthera tigris tigris*), and Indian leopard (*Panthera pardus fusca*) (Yelisetti et al. 2016).

As a result of the factors that influence the cell cycle synchronization, studies such as Yelisetti et al. (2016) and Wittayarat et al. (2012) described variability in the cell cycle arrest of four species of felids. Due to the variable effect and efficiency of each method of cell cycle arrest in different species, our study aimed to establish the most suitable protocol for cell G₀/G₁ arrest of northern tiger cat fibroblasts.

Materials and Methods

Chemicals and media

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and antibiotic-antimycotic solution were obtained from Gibco-BRL (Carlsbad, CA, USA). All the probes and other chemicals were purchase from Sigma-Aldrich (St. Louis, USA) or Invitrogen (Carlsbad, USA).

2527 *Bioethics, isolation, and primary culture of fibroblasts*

2528 All experiments were conducted according to the Ethics Committee of Animal Use of the Federal
 2529 Rural University of Semi-Arid (CEUA/UFERSA, no. 20/2023) and the Chico Mendes Institute
 2530 for Biodiversity Conservation (ICMBio, no. 88201-1). Three male and one female northern tiger
 2531 cats, approximately 3 years old, from zoos in Ceará, Brazil, were used for skin sample collection
 2532 (1.0–1.5 cm²).

2533

2534 The fragments were transported in DMEM containing 10% FBS and 2% antibiotic-antimycotic
 2535 solution at 4 °C for 4–5 h. In the laboratory, tissues were fragmented at 6.0 mm³ (3 mm × 2 mm ×
 2536 1 mm). The fragments were washed on DMEM supplemented with 10% FBS and 10% antibiotic-
 2537 antimycotic solution; 70% alcohol, and DMEM supplemented with 10% FBS and 2%
 2538 antibiotic-antimycotic solution (Viana et al.2025b). Next, four fragments were cultured on a dish
 2539 (60 mm ×15 mm) in DMEM with 10% FBS and 2% antibiotic-antimycotic solution at 38.5 °C
 2540 and 6.5% CO₂. After 16 days of cell cultured, they were disaggregated with 5 min of 0.25%
 2541 trypsin-EDTA, and submitted to the first passage.

2542 In the 3rd passage, fibroblasts were identified (Viana et al., 2025b) by immunofluorescence with
 2543 anti-vimentin. Subsequently, the resuspended fibroblasts were stored on cryovials with 10%
 2544 dimethyl sulfoxide, 10% FBS, and 0.2 M sucrose. Finally, fibroblasts were allocated overnight on
 2545 Mr. Frosty system® (Thermo Scientific Nalgene, Rochester, USA) in a cooling rate of 1°C/min,
 2546 next, storage on liquid nitrogen (-196°C).

2547

2548 *Thawing and fibroblast evaluations*

2549 Fibroblasts were thawed following the procedure described by Viana et al. (2025a). Briefly,
 2550 cryovials were kept 1 min at 25 °C and 4 min at 37 °C. Subsequently, cryoprotectants were

removed by exposure to DMEM with 10% FBS and 0.2 M sucrose per 15 min at 4 °C followed by centrifugation (600×g for 10 min). Next, cells were kept for 15 min at 25 °C with DMEM plus 10% FBS, followed by another centrifugation. Immediately after thawing the viability was measured by the cell membrane permeability to 0.4% trypan blue by the steps describes below.

Population Doubling Time (PDT) in hours and growth curve evaluated the ability of cell duplication and growth before the treatment. Initially, a 7.0×10^5 cells/mL concentration was distributed at seven wells on a 24-well plate (1.0×10^5 cells/mL per well) (Viana et al., 2025b). Each well was detached, and the concentration was counted every 24 h over 168 h. All the concentration counts were used in the growth curve determination and PDT measurement following the formula: $PDT = T \ln 2 / \ln (X_e / X_b)$, where T is the incubation time, X_b is the number of cells at the beginning of the incubation period, X_e is the number of cells at the end of the incubation period, and ln is the natural logarithm.

Cell treatments for G₀/G₁ arrest and experimental design

Three cell treatments for G₀/G₁ arrest were performed: chemical inhibition of CDK with RSV, serum starvation (0.5% FBS), and contact inhibition (Figure 1). The growing cells (GC) with 50–60% confluence was determined as the control group for all the treatments (Yelisetti et al. 2016). After all the treatments the cells were disaggregated with 5 min of 0.25% trypsin-EDTA and submitted to cell cycle and quality analysis.

After reaching a 60–70% confluence in the RSV, cells were exposed to RSV at 7.5 µM and 15 µM (Wittayarat et al. 2012). The effect of each concentration was evaluated after 24 and 48 h of treatment. The SS was performed by decreasing the FBS concentration to 0.5% (Młodawska et al.

2022). Cells with 60–70% confluence were kept in DMEM plus 0.5% of FBS for 24, 48, 72, and 96 h with medium change every 48 h (Rodrigues et al. 2023). After each treatment time, cells were detached and evaluated. In the CI, cells were cultured until 90–100% confluence. After reaching confluence, cells were analyzed after 24, 48, and 72 h of treatment to measure the effect of cell-to-cell contact inhibition at G₀/G₁ arrest (Yelisetti et al. 2016).

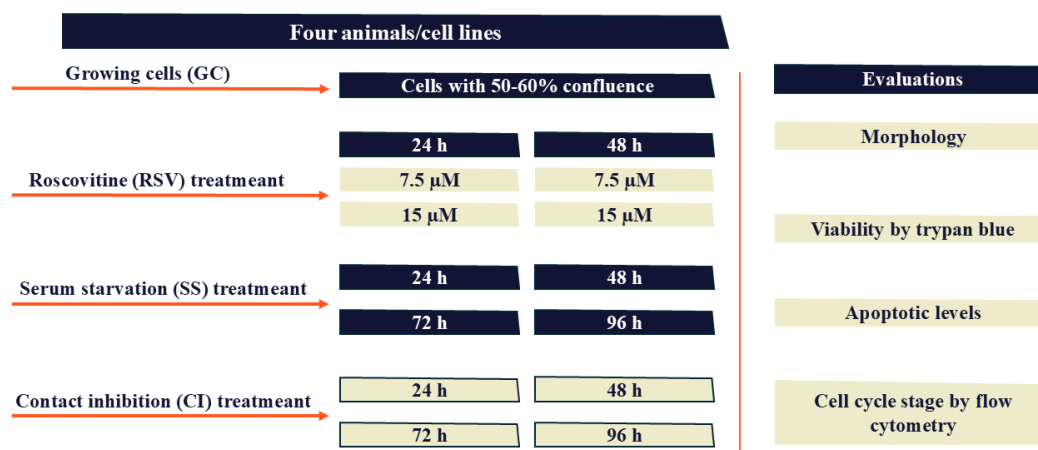


Figure 1. Experimental design of northern tiger cat fibroblasts treatments and analysis.

Cell cycle analysis

After all the treatments, cells were centrifuged at 600×g for 10 min and resuspended the pellet with 1.0 mL cooled at 70% ethanol for fixation and permeabilization. The cells were maintained for a maximum of 5 days at -20 °C. The ethanol was removed by rinsing in phosphate-buffered saline (PBS) and subsequently centrifuged at 400×g for 10 min. The cells were stained for 50 min at 4 °C with PBS plus 5 µg/mL propidium iodide (PI) and 50 µg/mL RNase (Yelisetti et al. 2016).

The different cell cycle levels were evaluated with a Guava EasyCyte flow cytometer (Guava

Technologies, Stamford, Lincolnshire, United Kingdom) among 15,000 events per sample. Graphics of red fluorescence vs. number of counts were generated (Figure 2) and taken to evaluate the cell percentage in the cell cycle phase (G_0/G_1 , S, G_2/M) and the sub- G_0/G_1 . Finally, the proportion of cells in each cell cycle phase and sub- G_0/G_1 was determined using MODFIT software version 5.0 (Verity, <https://www.vsh.com/products/mft/index.asp>) (Rodrigues et al. 2023).

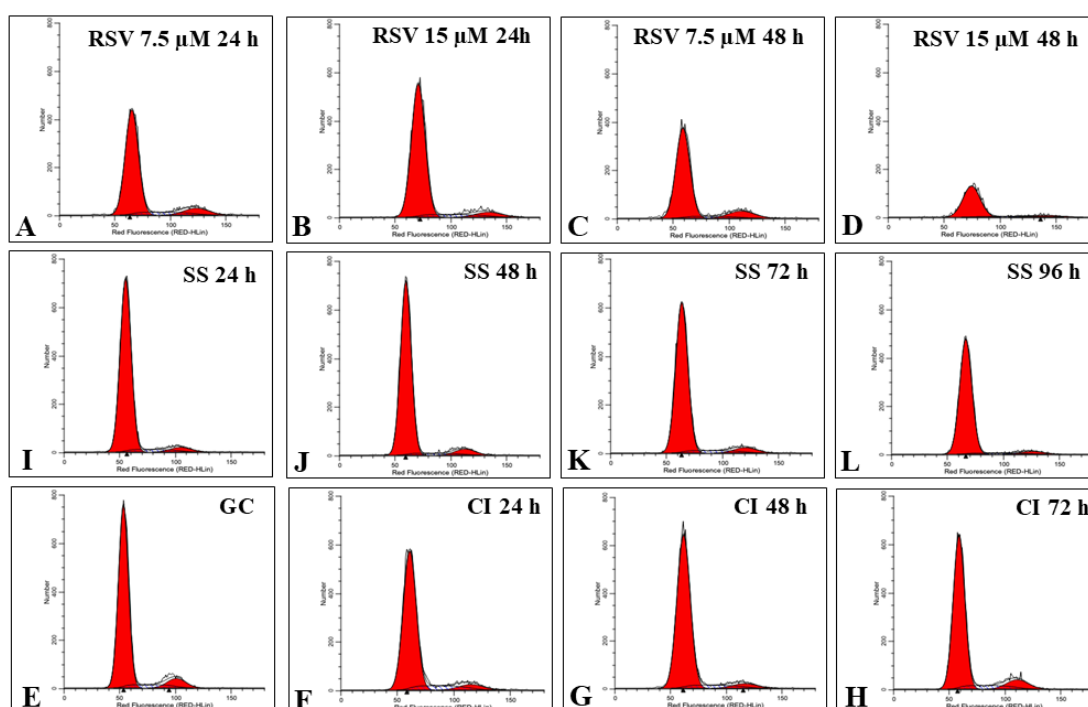


Figure 2. Representative histograms after analysis by Guava Easycyte flow cytometer of northern tiger cat fibroblasts subjected to RSV, CI and SS.

Morphology, viability, and apoptotic levels evaluation after G_0/G_1 arrest treatment

The cell morphology was analyzed with an inverted microscope (Leipzig IMx 400, PhoenixOptics, Germany). After each treatment, cytoplasmatic projection, cell size, cell detachment, and colony distribution were considered (Młodawska et al. 2022). The viability was measured by the cell membrane permeability to 0.4% trypan blue. The cells were counted in the

Neubauer chamber (Rodrigues et al. 2023). The translucent cells with intact membranes were counted as viable cells, and the blue cells with permeable membranes were classified as dead cells. The percentage of viable cells was determined by the viable cells/total cells $\times$ 100.

A fluorescence mixture of acridine orange (2 μ g/mL) and ethidium bromide (10 μ g/mL) in PBS was mixed on 50 μ L of cell suspension. The acridine orange bind the DNA and show a green fluorescence on live cells with regular chromatin condensation, and the dead cell were orange due the ethidium bromide incorporation (Nambiar and Hegde, 2016). At least 30 photographs were captured with a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at 20 $\times$ magnification (Veraguas et al. 2017). In each group, 300 cells were analyzed with Image J Software version 1.49 (National Institutes of Health, Bethesda, Maryland, USA). The cells can be classified as: I) uniform green color (Viable); II) non-uniform green color (Initial apoptotic); III) orange color (Late apoptotic); IV) red color (Necrosis).

Statistical analysis

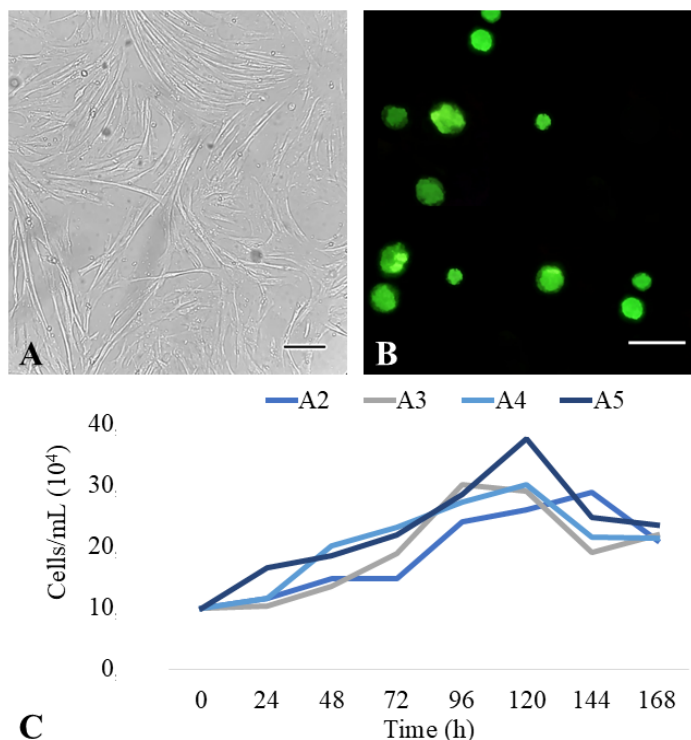
All the data were expressed as mean $\pm$ standard error (one animal/repetition) and analyzed using GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). The Shapiro-Wilk test verified normality, and Levene's test verified homoscedasticity. Since the data were not normally distributed, they were arcsine transformed and analyzed by ANOVA, followed by the Tukey test. Significance was set at $P < 0.05$.

Results

Immediately following cryopreservation, cells had a viability of approximately 60%, reaching a viability of $95.8\% \pm 1.4$ after culture. Subsequently 14 days of cell culture, cells showed a

2633 fusiform and regular shape, several cytoplasmatic projections, and central nucleus (Figure 3A).
 2634 Furthermore, cells showed a PDT of $21.5 \text{ h} \pm 1.5$ and a high number of viable cells after the
 2635 apoptosis evaluation ($99.8\% \pm 0.3$, Figure 3B). Additionally, the animals showed a standard
 2636 growth curve (Figure 3C) with clear phases of adaptation, exponential and stationary growth.

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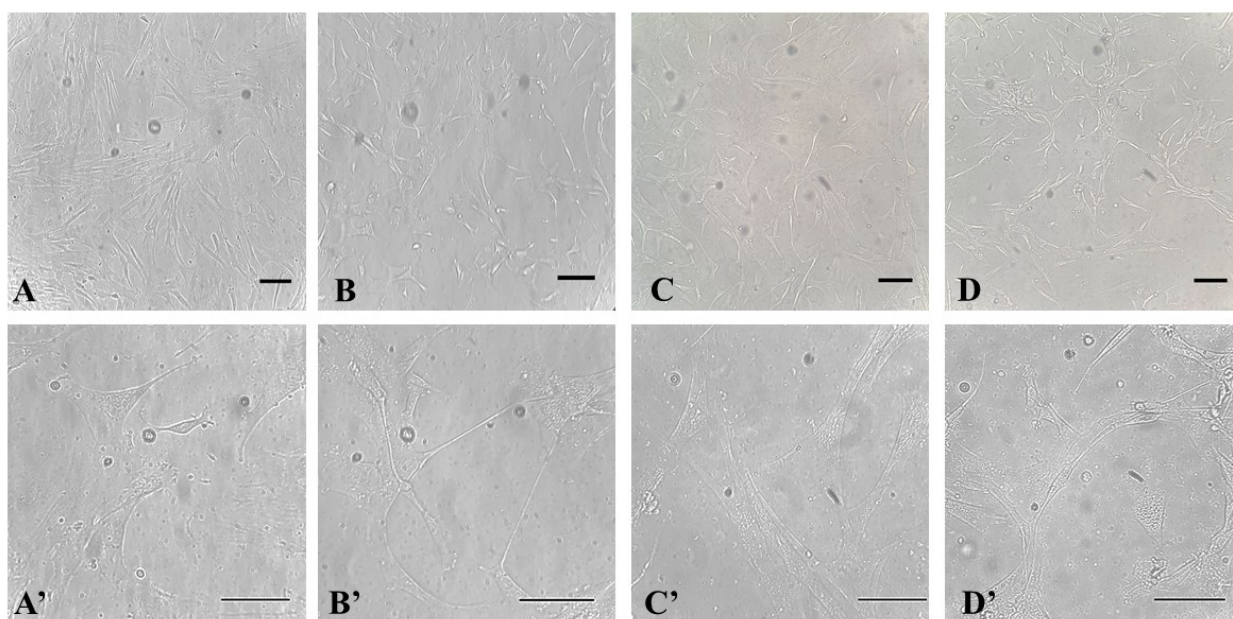
2639 **Figure 3.** Evaluation of northern tiger cat cells before treatment of cell arrest G_0/G_1 . **A)**
 2640 Morphology of fibroblasts with 50–60% of confluence; **B)** Apoptotic levels evaluation with
 2641 viable cells (green); **C)** Growth curve of each animal under 7 days (168 h) of cell culture. Scale
 2642 bar: $50 \mu\text{m}$; Magnification: $20\times$;

2643 *Response of northern tiger cat fibroblasts to RSV treatment*

2644 RSV treatment with $7.5 \mu\text{M}$ per 24 h (Figure 4A-A') or 48 h (Figure 4C-C') did not affect cell
 2645 morphology. On the other hand, the increase to $15 \mu\text{M}$ of RSV for 24 h (Figure 4B-B') and 48 h
 2646 (Figure 4D-D') decreased the cytoplasm size and projection and detached the cells from the dish.

2647 Even with morphology changes, the viability was not reduced (Figure 5A) after 24 h (RSV 7.5
 2648 μM : $84.7\% \pm 5.6$; RSV 15 μM : $92.9\% \pm 2.6$) or 48 h (RSV 7.5 μM : $89.7\% \pm 1.9$; RSV 15 μM :
 2649 $83.5\% \pm 5.5$).

2650



2651

2652 **Figure 4.** Morphology evaluation of northern tiger cat after RSV treatment; Magnification 20×:
 2653 **A)** RSV 7.5 μM 24 h; **B)** RSV 15 μM 24 h; **C)** RSV 7.5 μM 48 h; **D)** RSV 15 μM 48 h;
 2654 Magnification 40×: **A')** RSV 7.5 μM 24 h; **B')** RSV 15 μM 24 h; **C')** RSV 7.5 μM 48 h; **D')**
 2655 RSV 15 μM 48 h; Scale bar: 50 μm ;

2656

2657 In the percentage of apoptotic levels (Figure 5B-E), only the RSV at 7.5 μM for 24 h ($94.3\% \pm$
 2658 2.8) kept the percentage of viable cells of GC ($99.8\% \pm 0.3$, Figure 5F). In contrast, the other
 2659 groups with RSV 15 μM for 24 h ($91.5\% \pm 0.8$), 7.5 μM for 48 h ($89.0\% \pm 3.7$), and 15 μM for
 2660 48 h ($90.0\% \pm 1.7$) had viability decreased. The toxic effect of RSV was observed at initial
 2661 apoptotic cells, where all the groups increased the values of $0.3\% \pm 0.3$ to around 6%.
 2662 Alternatively, most groups did not affect the late apoptotic cells by around 1%, except the group
 2663 RSV 48 h 7.5 μM , which increased to $3.5\% \pm 0.6$. RSV did not affect necrotic cells, remaining

around 0.5%.

Based on the analysis of cell cycle stage graphics (Figure 2A-D), RSV at 24 h or 48 h, regardless of concentration, maintains the number of G₀/G₁ at approximately 85% (Table 1). Similarly, all the RSV treatments preserved the proportion of the G₂/M phase. In contrast, the treatment of RSV for 48 h with 7.5 μ M or 15 μ M increased in the S phase to 8%. Notably, none of the RSV treatments, at any time or concentration, increased the sub-G₀/G₁ population, indicating an absence of apoptosis.

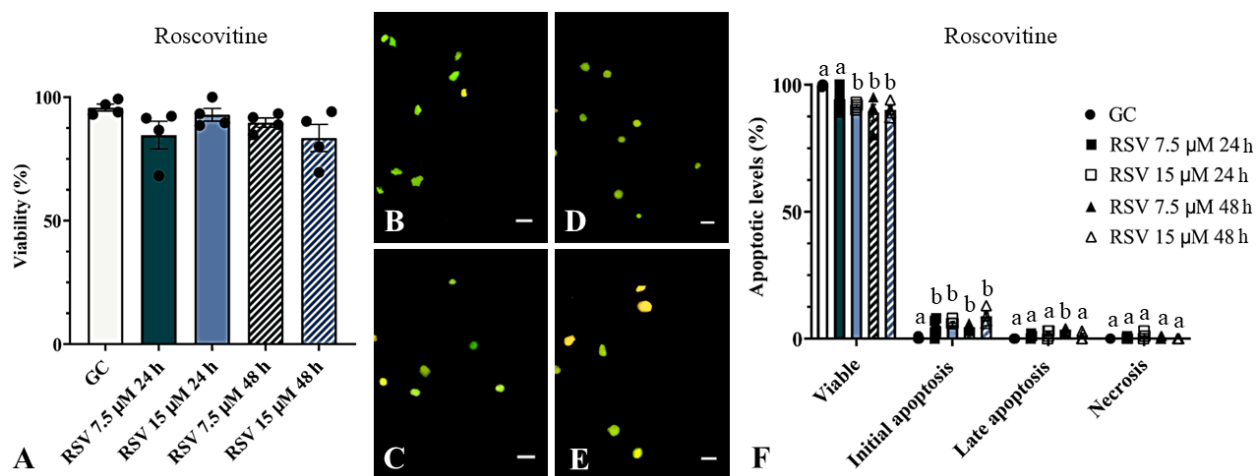


Figure 5. Evaluation of viability and apoptotic levels of northern tiger cat after RSV treatment; A) Viability measurement by trypan blue assay; Evaluation of apoptotic levels with acridine orange and ethidium bromate: B) RSV 7.5 μ M 24 h; C) RSV 15 μ M 24 h; D) RSV 7.5 μ M 48 h; E) RSV 15 μ M 48 h; F) Percentage of apoptotic levels Scale bar: 50 μ m; ^{a,b}: values with different superscript represent significant differences (P < 0.05).

Table 1. Effect of concentration and time of exposure of RSV on the percentage of northern tiger cat fibroblasts in the G₀/G₁, S, and G₂/M phases of the cycle.

Treatment	Cell cycle phase (%)			
	G ₀ /G ₁	S	G ₂ /M	Sub G ₀ /G ₁
GC	86.0 ± 0.9 ^a	6.3 ± 0.5 ^a	7.8 ± 0.7 ^a	0.0 ± 0.0 ^a
RSV 7.5 µM 24 h	83.6 ± 1.2 ^a	7.2 ± 0.7 ^a	9.1 ± 0.6 ^a	0.0 ± 0.0 ^a
RSV 15 µM 24 h	85.2 ± 3.7 ^a	6.4 ± 1.4 ^a	8.5 ± 2.5 ^a	0.0 ± 0.0 ^a
RSV 7.5 µM 48 h	84.4 ± 1.7 ^a	8.5 ± 0.7 ^b	7.1 ± 1.1 ^a	0.0 ± 0.0 ^a
RSV 15 µM 48 h	81.1 ± 1.5 ^a	8.0 ± 0.3 ^b	10.9 ± 1.5 ^a	0.0 ± 0.0 ^a

GC: growing cells; RSV: roscovitine; values expressed as mean ± standard error; ^{a,b}: values with different superscript represent significant differences (P < 0.05).

Response of northern tiger cat fibroblasts to SS treatment

The morphology of cells was altered after increasing the treatment time (Figure 6A-D). At the first 24 h (Figure 6A'), cells showed a regular morphology, a large cytoplasm, and several cytoplasm projections. After 24 h, the morphology changed to a thinner cytoplasm with less cell projection, keeping the fusiform shape and the length of the cells (Figure 6B'-D').

In contrast, the viability of the cells was kept at 82.5% ± 3.1 until 90.9% ± 1.6 (Figure 7A). On the other hand, the number of viable cells was affected at 24 h (93.3% ± 0.6), 48 h (92.8% ± 2.0), 72 h (94.0% ± 2.5), and 96 h (95.5% ± 1.5) of treatment (Fig. 7 B-F). Moreover, the number of initial apoptotic cells increased only in the 48-h treatment (4.8% ± 1.4), whereas all the other treatment times remained around 2%. Moreover, the late apoptotic cells were increased to 2% in almost all the groups except for 96 h (1.3% ± 0.3). Finally, the percentage of necrotic cells was kept between 0.3 ± 0.3 and 0.8 ± 0.6.

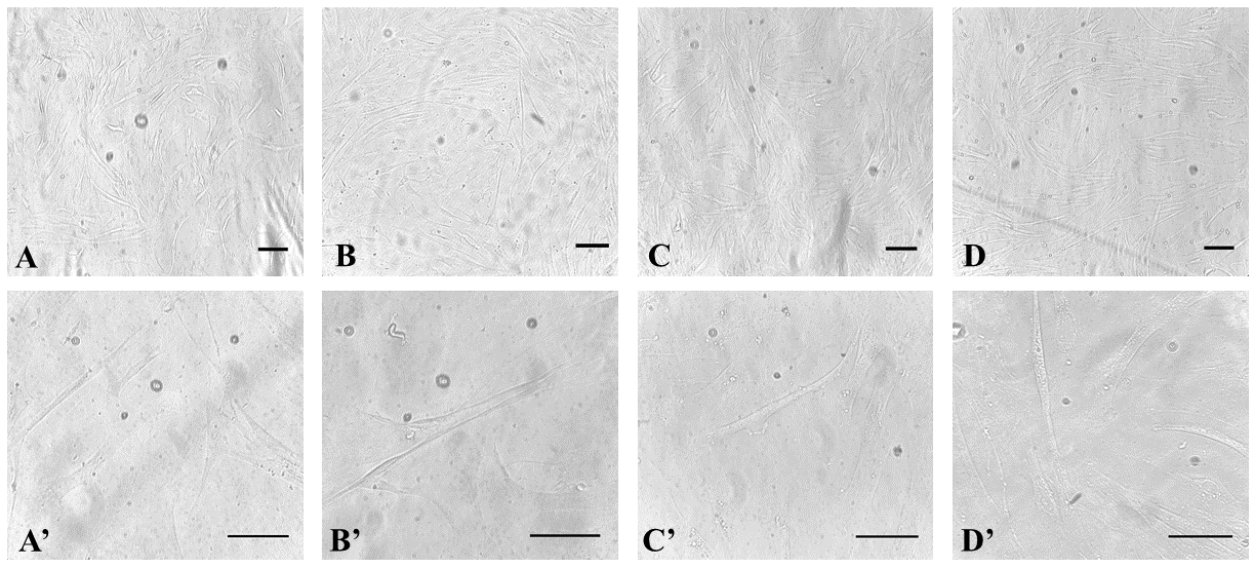


Figure 6. Morphology evaluation of northern tiger cat cells after SS treatment; Magnification 20×: **A)** SS 24 h; **B)** SS 48 h; **C)** SS 72 h; **D)** SS 96 h; Magnification 40×: **A')** SS 24h; **B')** SS 48 h; **C')** SS 72 h; **D')** SS 96 h Scale bar: 50 µm;

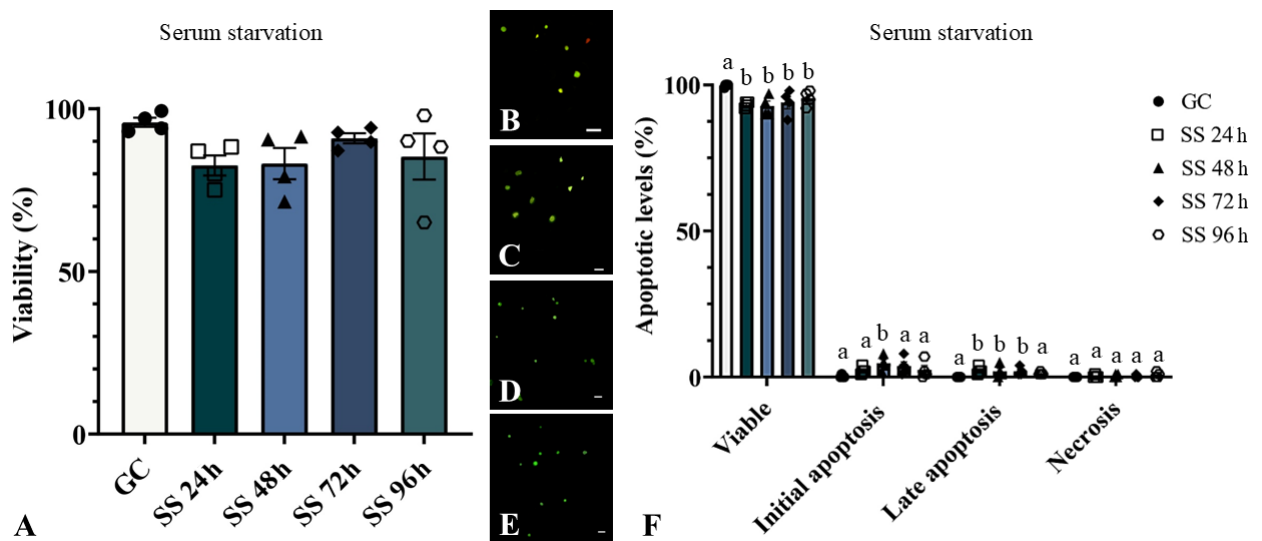


Figure 7. Evaluation of viability and apoptotic levels of northern tiger cat after SS treatment; **A)** Viability measurement by trypan blue assay after SS; Evaluation of apoptotic levels with acridine orange and ethidium bromate: **B)** SS 24 h; **C)** SS 48 h; **D)** SS 72 h; **E)** SS 96 h; **F)** Percentage of viable, initial apoptotic, late apoptotic and necrotic cells. Scale bar: 50 µm; ^{a,b}: values with

different superscript represent significant differences ($P < 0.05$).

In the cell cycle phase analysis (Table 2, Figure 2E-H), 24 h of SS increased the proportion of G_0/G_1 from $86.0\% \pm 0.9$ to $91.4\% \pm 1.7$, while the other groups kept around 86–90%. All the treatments decreased the initial number of $6.3\% \pm 0.5$ at the S phase to 4%, similarly for the G_2/M population, which reduced from 7.8 ± 0.7 to around 5%. Finally, the sub- G_0/G_1 proportion was not affected by the treatment.

Table 2. Effect of SS on the percentage of northern tiger cat fibroblasts in the G_0/G_1 , S, and G_2/M phases of the cycle.

Treatment	Cell cycle phase (%)			
	G_0/G_1	S	G_2/M	Sub G_0/G_1
GC	86.0 ± 0.9^b	6.3 ± 0.5^b	7.8 ± 0.7^b	0.0 ± 0.0^a
SS 24 h	91.4 ± 1.7^a	4.4 ± 1.0^a	4.3 ± 0.9^a	0.0 ± 0.0^a
SS 48 h	90.8 ± 0.7^b	4.3 ± 0.4^a	4.9 ± 0.5^a	0.0 ± 0.0^a
SS 72 h	88.8 ± 1.3^b	5.7 ± 0.7^a	5.5 ± 0.7^a	0.0 ± 0.0^a
SS 96 h	89.5 ± 1.2^b	4.7 ± 0.4^a	5.8 ± 0.9^a	0.0 ± 0.0^a

GC: growing cells; SS: serum starvation; values expressed as mean $\pm$ standard error; ^{a,b}: values with different superscript represent significant differences ($P < 0.05$).

Response of northern tiger cat fibroblasts to CI treatment

The CI treatment did not affect the cells (Figure 8A-C). Cells maintained the fusiform shape and the cytoplasm size. Also, cell overlapping can be seen due to confluency (Figure 8A'-C'). Similarly, viability was kept after 24 h ($95.6\% \pm 1.5$), 48 h ($95.5\% \pm 1.4$), and 72 h ($94.7\% \pm 1.8$)

of CI (Figure 9A). Furthermore, the number of viable cells ($\sim 98\%$), initial apoptotic cells ($\sim 1\%$), late apoptotic ($\sim 0.5\%$), and necrosis ($\sim 0.2\%$) cells were preserved (Figure 9B-E).

Finally, CI did not change the proportion of the G_0/G_1 phase, maintained around 85% , similarly for the G_2/M phase, which was kept between $7.8\% \pm 0.7$ and $8.3\% \pm 0.5$ (Table 3). On the other hand, the proportion of S increased after 24 h ($10.1\% \pm 1.5$), 48 h ($7.2\% \pm 0.5$), and 72 h ($7.1\% \pm 0.4$). Even under stress, the sub- G_0/G_1 proportion was preserved at 0% .

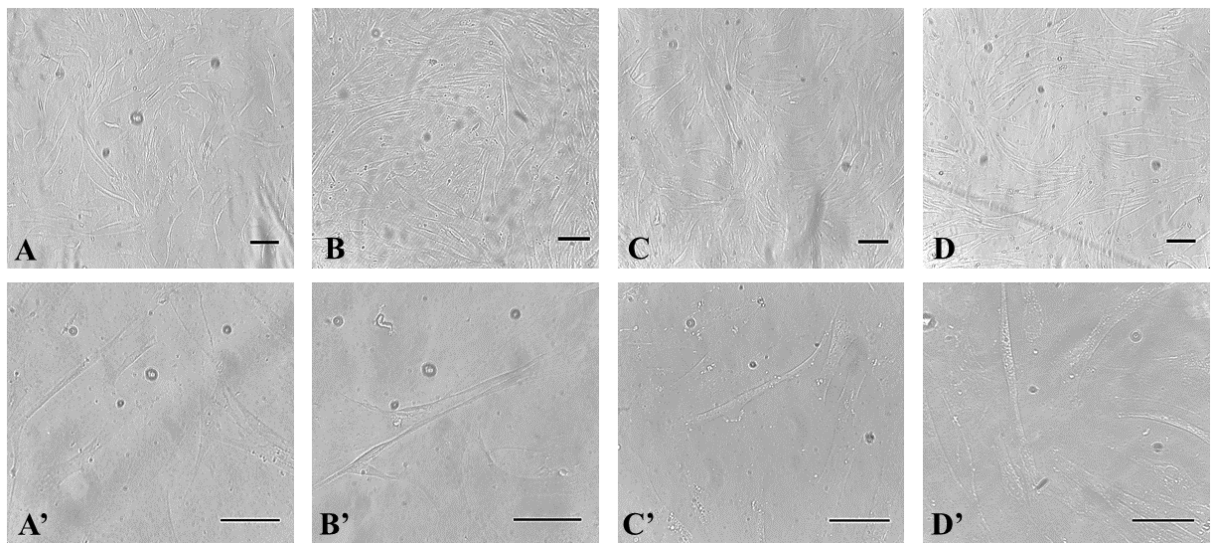


Figure 8. Morphology evaluation of northern tiger cat after CI treatment; Magnification 20 $\times$: **A)**

CI 24h; **B)** CI 48h; **C)** CI 72h; Magnification 40 $\times$: **A')** CI 24h; **B')** CI 48h; **C')** CI 72h; Scale bar:

50 μm ;

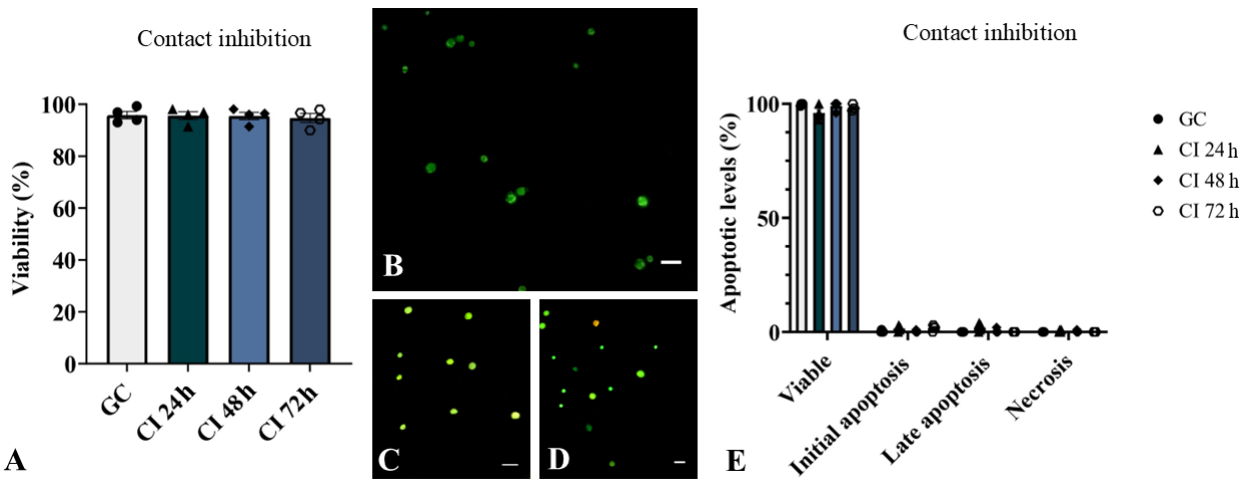


Figure 9. Evaluation of viability and apoptotic levels of northern tiger cat after CI treatment; **A)** Viability measurement by trypan blue assay; Evaluation of apoptotic levels with acridine orange and ethidium bromate: **B)** CI 24h; **C)** CI 48h; **D)** CI 72h; **F)** Percentage of viable, initial apoptotic, late apoptotic and necrotic cells. Scale bar: 50 μ m; absence of superscript represents no significant differences ($P > 0.05$).

Table 3. Effect of CI on the percentage of northern tiger cat fibroblasts in the G_0/G_1 , S, and G_2/M phases of the cycle.

Treatment	Cell cycle phase (%)			
	G_0/G_1	S	G_2/M	Sub G_0/G_1
GC	86.0 ± 0.9^a	6.3 ± 0.5^a	7.8 ± 0.7^a	0.0 ± 0.0^a
CI 24 h	84.0 ± 1.8^a	10.1 ± 1.5^b	6.0 ± 0.4^a	0.0 ± 0.0^a
CI 48 h	87.3 ± 1.4^a	7.2 ± 0.5^b	5.5 ± 0.9^a	0.0 ± 0.0^a
CI 72 h	84.7 ± 0.8^a	7.1 ± 0.4^b	8.3 ± 0.5^a	0.0 ± 0.0^a

GC: growing cells; CI: contact inhibition; values expressed as mean $\pm$ standard error; ^{a,b}: values with different superscript represent significant differences ($P < 0.05$).

4. Discussion

The G₀/G₁ cell cycle arrest is an essential step for producing SCNT embryos (Gómez et al., 2004) and iPSC (Chen et al., 2012), directly influencing the success of these techniques. However, obtaining the most efficient cell arrest protocols varies among protocols and species (Młodawska et al., 2022), making it necessary to balance cell viability and effective cell arrest.

Our study identified the most effective protocol for promoting cell arrest in northern tiger cat cells. RSV was less effective on cell arrest and more toxic to cells, especially at a concentration of 15 µM. Inversely, CI treatment for 24–72 h was not toxic but also not effective at increasing G₀/G₁ arrest. Finally, SS treatment was the most effective, significantly increasing G₀/G₁ arrest after 24 h, although it also reduced cell viability.

RSV reduced cytoplasm extension and the size of northern tiger cat cells. Similarly, in human fibroblasts, Steinman et al. (2012) described that RSV decreases the expression of collagen and fibronectin, the main components of the extracellular matrix. Additionally, RSV inhibits CDKs, which can affect the fibroblast pathways. For example, CDK 1 supports cell adhesion and extracellular matrix expression (Kamranvar et al, 2022). Consequently, the combined effect of RSV on extracellular matrix and CDK 1 inhibition can change cell morphology.

Furthermore, RSV increased apoptotic levels and reduced the viable cell number. Interestingly, in other felid species, such as marbled cats and Asian golden cats, increasing the RSV concentration from 7.5 µM to 15 µM did not affect apoptotic levels (Wittayarat et al., 2012). This shows that northern tiger cat cells may be more susceptible to RSV damage. RSV was not effective in

inducing G₀/G₁ arrest in some species, such as leopard (*Panthera pardus*) at 7.5 µM or 15 µM (Wittayarat et al., 2012) and puma at 15 µM for 24 h (Rodrigues et al., 2023) and in this study. Due to the damage to northern tiger cat cell viability, morphology, and the inefficiency of RSV, other successful chemicals, such as sodium butyrate (Yelisetti et al., 2016), can be explored.

The CI method of G₀/G₁ cell arrest was ineffective on northern tiger cats, with no changes in cell viability and morphology. In successful CI, the DNA synthesis is suspended, cadherin levels decrease, and the Yes-associated protein (YAP)-1 is inhibited (McClatchey & Yap, 2012). Cell characteristics can influence the required time for the CI effect, including cell shape, colony growth, and cell cycle speed (Schnyder et al., 2020). For instance, in asian gold cats and leopards, 5 days of CI improved G₀/G₁ (~88%), while in jaguarundi (*Puma yagouaroundi*), 5 days increased the G₀/G₁ and dead cells (Młodawska et al., 2022). In this context, increasing the treatment time can be the one solution to increase the G₀/G₁ of northern tiger cats after CI.

Gomez et al. (2004) used the SS method to obtain the first birth cloned wild felid, the african wildcat (*Felis silvestris lybica*), and it has since been implemented in several species. In northern tiger cat fibroblasts, SS treatment changed cell morphology. Similarly, Veraguas et al. (2017) showed a decrease in kodkod (*Leopardus guigna*) cell size after SS treatment. Curiously, smaller cells in pigs can improve SCNT's success (Jiao et al., 2021). Therefore, the reduction in cell size following SS treatment in northern tiger cats could influence the efficiency of future SCNT development.

Compared to other methods for cell cycle arrest, SS after 24 h was the only one to increase the G₀/G₁ population proportion, however, with a slight viable decrease. SS treatment after 24 h has

been effective in various wild felid species, such as Asian golden cats (Wittayarat et al., 2012) and manul (*Felis manul*) (Młodawska et al., 2022). Furthermore, in the Indian leopard and Indian lion (Yelisetti et al., 2016), similar to the northern tiger cat, SS treatment decreased cell viability. However, it did not interfere with SCNT embryo production. Therefore, further research is necessary to analyze the impact of these effects on SCNT production for northern tiger cats.

5. Conclusions

In summary, our study demonstrated that RSV treatment had no significant impact on the G₀/G₁ phase; however, it increased cellular damage to northern tiger cat cells. Similarly, CI treatment was ineffective in enhancing G₀/G₁ arrest and did not affect cell viability. In contrast, SS treatment for 24 h improved G₀/G₁ arrest but caused a slight decrease in cell viability. These findings highlight the need for further optimization of cell cycle G₀/G₁ arrest protocols for northern tiger cats, including exploring combinations of techniques and alternative chemical treatments. Additionally, studies should investigate whether the damage caused by SS treatment impacts embryo production and the subsequent applications of these cells.

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Conflict of interest

The authors declare having no conflict of interest.

Funding Declaration

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Data availability

The data used to support the findings of this study are included within the article.

Author contribution declaration

JVS Viana: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original draft. **BS Barbosa:** Methodology. **LLV Rodrigues:** Methodology. **ABM Pereira:** Methodology. **PV Alves:** Methodology. **HVR Silva:** Methodology. **SSA Maranhão:** Methodology. **CRK Paier:** Methodology. **MCS Luciano:** Methodology. **C Pessoa:** Methodology. **AF Pereira:** Conceptualization, Formal Analysis, Investigation, Resources, Data Curation, Writing – Review & Editing, Supervision, Funding acquisition.

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CAPÍTULO 6- CONSIDERAÇÕES FINAIS

CONCLUSÕES

O trabalho foi desenvolvido com a premissa de estabelecer as etapas iniciais visando a aplicação de células somáticas de gato-do-mato-pequeno. Inicialmente, foram estabelecidas as condições de cultivo *in vitro*, células foram caracterizadas e analisadas quanto aos efeitos do cultivo prolongado e a capacidade de criopreservação das células. Posteriormente, tomando como base estudos anteriores, nós visamos otimizar a solução de criopreservação e os protocolos de sincronização das células em G₀/G₁.

Portanto, nós concluimos que células isoladas da região auricular do gato-do-mato-pequeno foram identificadas como fibroblastos e cariótipo normal. Além disso, células sofreram danos ocasionados pelo cultivo *in vitro* a partir da sétima até a décima primeira passagem. Ainda, na primeira etapa foi observada necessidade otimização dos protocolos de criopreservação para espécie.

Desse modo, no segundo experimento foi observada que a adição de sacarose e L-prolina não resultou em melhoria na qualidade celular após a criopreservação quando comparado aos grupos na ausência de crioprotetores extracelulares. Por outro lado, o aumento da concentração de DMSO de 2,5% para 10% promoveu uma melhor recuperação celular após criopreservação. Finalmente, no terceiro experimento, quanto às metodologias de sincronização, o inibidor químico roscovitina e a inibição por contato não foi efetiva no aumento da taxa de células em G₀/G₁. Desse modo, a privação de soro fetal bovino por 24 h foi o único método eficiente nessa etapa, mesmo ocasionando leve redução na viabilidade celular.

PERSPECTIVAS

Nós estabelecemos os primeiros passos no entendimento das etapas iniciais relacionadas as células somáticas de gato-do-mato-pequeno, dando assim mais um passo para as próximas aplicações, estudos e conservação da espécie. Devido aos efeitos do número de passagens sobre as células somáticas de gato-do-mato-pequeno, estratégias voltadas para imortalização celular e otimização de suplementações para meio de cultivo *in vitro* podem ser estudadas visando contornar as injúrias observadas e uma melhor aplicação dessas células.

Adicionalmente, testar soluções de criopreservação buscando reduzir ou substituir o DMSO podem ser estudadas, como a utilização do etilenoglicol ou de soluções crioprotetoras na ausência de DMSO. Finalmente, visando otimizar os protocolos de sincronização para espécie, ainda poderiam ser averiguadas a combinação de técnicas como a inibição por contato com privação de soro fetal bovino, ou inibidores químicos alternativos como o butirato de sódio.

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ANEXOS

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ANEXO No. 1: ARTIGO “ESTABLISHMENT AND CHARACTERIZATION OF
FIBROBLAST LINES FROM THE NORTHERN TIGER CAT (*Leopardus tigrinus*,
SCHREBER, 1775) DURING EXTENDED PASSAGE AND CRYOPRESERVATION”
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RESEARCH ARTICLE

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Establishment and characterization of fibroblast lines from the northern tiger cat (*Leopardus tigrinus*, Schreber, 1775) during extended passage and cryopreservation

João Vitor da Silva Viana¹ | Lhara Ricarlany Medeiros de Oliveira¹ |
Luanna Lorena Vieira Rodrigues¹ | Yara Letícia Frutuoso e Silva¹ |
Ana Livia Rocha Rodrigues¹ | Alexandre Rodrigues Silva² |
Patrícia Vasconcelos Alves³ | Herlon Victor Rodrigues Silva⁴ |
Alexsandra Fernandes Pereira¹

¹Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoro, Brazil

²Laboratory of Animal Germplasm Conservation, UFERSA, Mossoro, Brazil

³Sargento Prata City Zoo, Fortaleza, Brazil

⁴Laboratory of Carnivore Reproduction, State University of Ceará, Fortaleza, Brazil

Correspondence

Alexsandra Fernandes Pereira, Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Av. Francisco Mota, 572, Mossoró, Brazil.
Email: alexsandra.pereira@ufersa.edu.br

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Abstract

The establishment of fibroblast lines enables several applications from the formation of biobanks for the conservation of biodiversity to the use of these cells in physiological and toxicological assays. Considered a species vulnerable to extinction, the characterization of fibroblastic lines of northern tiger cat would contribute to its conservation. Therefore, we established and characterized fibroblasts derived from northern tiger cat during extended passage (third, seventh, and eleventh passages) and cryopreservation with regard to the morphology, viability, apoptotic classification, metabolism, proliferative activity, and oxidative stress by reactive oxygen species (ROS) levels and mitochondrial membrane potential ($\Delta\Psi_m$). Initially, we identified four dermal fibroblast lines by morphology, immunophenotyping, and karyotyping assays. *In vitro* culture after the third, seventh, and eleventh passages did not affect the viability, apoptotic classification, and ROS levels. Nevertheless, cells at seventh and eleventh passages featured a reduction in metabolism and an alteration in $\Delta\Psi_m$ when compared to third passage cells. Additionally, cells at eleventh passage showed changes in the proliferative activity and morphology when compared to other passages. Regarding cryopreservation, no effect was observed on cryopreserved cells for morphology, viability, apoptotic classification, metabolism, and proliferative activity. Nevertheless, cryopreserved cells had alteration for ROS levels and $\Delta\Psi_m$. In summary, fibroblasts from northern tiger cat were affected by extended passage (seventh and eleventh passages) and cryopreservation. Adjustments to the *in vitro* culture and cryopreservation are necessary to reduce cellular oxidative stress caused by *in vitro* conditions.

KEYWORDS

cell line, cryobank, *in vitro* culture, senescence, small wild felids

1 | INTRODUCTION

The northern tiger cat (*Leopardus tigrinus*, Schreber, 1775) is a small wild cat found in South and Central America, in countries such as Argentina, Venezuela, Paraguay, Costa Rica, and Brazil (Jarretta et al., 2004). This species plays a fundamental role in the food chain as a mesopredator, influencing the control of its prey and, consequently, the diversity of the flora (Alberti et al., 2023). Moreover, it has been studied due to its scarce information (Oliveira et al., 2020), lower population density caused by the presence of large felids (Fox-Rosales & de Oliveira, 2023), and classification as vulnerable to extinction (Payan & Oliveira, 2016).

Given this scenario, several conservation strategies have been implemented for the northern tiger cat with a focus on *in situ* (Payne et al., 2024), *ex situ in vivo* (Futas et al., 2022), and *ex situ in vitro* (Angrimani et al., 2017) methodologies. In this last group, protocols were developed for sperm recovery (Juvenal et al., 2008), and cryopreservation (Baudi et al., 2008), ovarian tissue *in vitro* culture and cryopreservation (Wiedemann et al., 2013), oocyte recovery (Carreiro et al., 2017), and somatic cell cryopreservation (Arantes et al., 2021), aiming at the formation of cryobanks. With respect to somatic cells, Arantes et al. (2021) were pioneers in the isolation and cryopreservation of somatic cells from these animals, evaluating different slow freezing conditions. Although the results are promising, the authors suggest optimizing these conditions for adequate conditioning of these cells.

The optimization of these protocols depends on the establishment of cell lines through identification of type and genetic variations (Liu et al., 2010), cell characterization during extended passage (Silva et al., 2021), and assessment of damage caused by cryopreservation (Arantes et al., 2021). In wild felids, skin-derived fibroblasts have been the cells of choice for conservation from these biobanks aiming at nuclear reprogramming (Verma et al., 2012). In this sense, an important point is the cellular senescence, which has been variable, being observed at the fourth passage for the fishing cat (*Prionailurus viverrinus*, Sukparangsi et al., 2022), or tenth passage for the puma (*Puma concolor*, Lira et al., 2022). Additionally, the evaluation of the cell passages is a tool for understanding not just the effect on the reprogramming but also the supplementation of medium (Sukparangsi et al., 2022).

Moreover, associated with the number of passages, the assessment of the damage caused by cryopreservation allows understanding the cellular behavior in the face of stressful conditions (Lira et al., 2022). In general, the cryopreservation can cause physical and biochemical damage on cell metabolism (Murray & Gibson, 2022). Therefore, the evaluation of different cellular quality parameters permits knowing more precisely the cellular response to these conditions (Baust et al., 2017).

Finally, the application of artificial reproductive technologies (ARTs) is directly dependent on the first steps of establishment and characterization of cell lines, and the effects of the cryopreservation over several steps (Thongphakdee et al., 2020). Therefore, we aimed to establish and characterize fibroblasts derived from northern tiger

cat during extended passage (third, seventh, and eleventh passages) and cryopreservation with regard to the morphology, viability, apoptotic classification, metabolism, proliferative activity, and oxidative stress. Additionally, we expected to understand and establish the minimum conditions for the formation of cryobanks for the species, contributing to conservation.

2 | MATERIALS AND METHODS

2.1 | Chemical and media

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and antibiotic-antimycotic solution were obtained from Gibco-BRL (Carlsbad, CA, USA). The anti-vimentin antibody and goat anti-mouse IgG (Alexa Fluor® 488) were purchased from Abcam (Cambridge, MA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

2.2 | Animals care and skin collection

All the experiments were approved by the Ethics Committee of Animal Use of the Federal Rural University of Semi-Arid (CEUA/UFERSA, no. 20/2023) and the Chico Mendes Institute for Biodiversity Conservation (no. 88201-1; ICMBio). Three males and one female with ages between 2 and 3 years, located in zoos in cities of Ceara, Brazil, were used for skin recovery.

Ear skin samples (1.0–1.5 cm²) were collected using surgical scissors after administration of 0.04 mg/kg dexmedetomidine (Dexdormitor®, Zoetis, Campinas, SP, Brazil) combined with 5 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, São Paulo, SP, Brazil) and mechanical containment (Juvenal et al., 2008). The tissues were transported to the laboratory in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution at 4°C for 4–5 h.

2.3 | Establishment of primary culture, cell isolation and experimental design

In the laboratory, fragments were trichotomized, fragmented in 6.0 mm³ (3 × 2 × 1 mm), and washed according to the sequence of media: (i) DMEM supplemented with 10% FBS and 10% antibiotic-antimycotic solution; (ii) 70% alcohol, and (iii) DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution (Lira et al., 2022). Subsequently, all fragments (four or five per dish per animal) were cultured in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution at 38.5°C and 6.5% CO₂.

Monitoring of the primary culture occurred daily using an inverted microscope (Leipzig IMx 400, PhoenixOptics, Germany) with total change of medium. Tissues were evaluated for day and percentage of attachment, detachment, and subconfluence, duration of culture, and absence of bacteria and fungi. When the cells reached

70% confluence, tissues were removed and cells were trypsinized with 0.25% trypsin-EDTA solution for the first passage (Lira et al., 2022).

The study was divided into three experiments. In the first experiment, cells were grown under primary culture conditions, and when cells reached the third passage were analyzed regarding morphology, confirming the cell type using immunofluorescence assay, karyotyping, and contamination absence. In the second experiment, cells were cultured and evaluated after the third, seventh, and eleventh passages with respect to the effects of the culture on the morphology, viability, apoptotic classification, metabolism, proliferative activity, and oxidative stress by reactive oxygen species (ROS) levels, and mitochondrial membrane potential ($\Delta\Psi_m$). In the third experiment, cryopreserved cells were evaluated using the aforementioned parameters and ultrastructure. The characteristics of the cryopreserved cells were compared with those of non-cryopreserved cells.

2.4 | Morphology and immunofluorescence for cell type confirmation

Initially, cells were evaluated for morphological characteristics by observation of cytoplasmic extensions, shape and cell surface using inverted microscope (Gómez et al., 2008). For immunofluorescence, cells were fixed with 4% paraformaldehyde for 15 min and, subsequently, washed with chilled phosphate-buffered saline (PBS). Cells were incubated with antigen retrieval (100 mM Tris, 5% urea, pH 9.5), permeabilized for 1 h with 0.4% Triton X-100, and incubated with blocking solution (PBS with 5% BSA and 0.1% Tween 20, Silva et al., 2021). Finally, an immunoassay was carried out for 24 h at 4°C with mouse anti-vimentin antibody (ab89779, 1:200) and then cells were incubated with the secondary antibody (goat anti-mouse IgG, Alexa Fluor® 488, ab150113, 1:400) for 1 h at 25°C in the dark. The cell nucleus was identified by the incubation with 1 µg/mL Hoechst 33342 for 1 min; the cytoplasmic extensions were observed in a green fluorescence and the nucleus in a blue fluorescence using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan), with 20× magnification.

2.5 | Karyotype analysis

Cells were evaluated by karyotype according to Song et al. (2007), with modifications. Cells with 70% confluence were incubated with 0.1% colchicine for 6 h at 38.5°C and 6.5% CO₂ for the metaphase synchronization. After this period, cells were trypsinized, the pellet was resuspended in 5 mL hypotonic solution (0.075 M KCl), maintained for 10 min at 37°C and centrifuged (600 × g for 10 min) after incubation. Cells with ruptured membrane and exposed chromosomes were fixed in 5 mL methanol and acetic acid solution, followed by centrifugation (600 × g for 10 min). For the visualization of chromosome structure, fixed chromosome solution was dropped onto slides, dried at 25°C and stained for 5 min with 5% Giemsa solution.

The Giemsa solution excess was washed with distilled water and the slides were dried for visualization at 100× magnification on light microscope (Olympus BX51TF, Tokyo, Japan) and chromosome analysis. The final image of the chromosomes with the optimal staining and definition was selected. The karyotype was arranged based on the Eroğlu (2017) description, using the software GNU Image Manipulation Program (GIMP) version 2.10.22.

2.6 | Evaluation of contamination absence

The absence of contamination was assessed by incubating 1.0 mL cell suspension in a 35 × 10 mm well-dish containing a nutrient medium (DMEM with 10% FBS) devoid of antibiotic-antimycotic solution (Lira et al., 2022). The medium was replaced daily, and the cells were examined under an inverted microscope to detect any bacterial and fungal contamination. Additionally, the medium color and opacity were monitored for any changes.

2.7 | Cell cryopreservation

Cells at the third passage were trypsinized, counted and allocated in cryovials (1.0 × 10⁵ cells/mL) with DMEM supplemented with 10% FBS and 2.5% dimethyl sulfoxide (DMSO) (Arantes et al., 2021). Cells were exposed to cryopreservation solution, allocated in Mr. Frosty freezing container for 15 min at 4°C. Subsequently, cells were stored overnight at -80°C freezer with a cooling rate of 1°C/min. Finally, cells were stored in liquid nitrogen (-196°C).

After 2 weeks, cells were thawed in a graduate temperature elevation with exposition for 1 min at 25°C and immersion in water bath for 4 min at 37°C. The removal of cryoprotectants was performed twice through dilution with DMEM associated with 10% FBS followed by centrifugation (600 × g for 10 min, Rodrigues et al., 2023). Finally, cells were kept for 15 min at 25°C and cultured in dish for the analysis. Cryopreserved cells were evaluated for the cell quality parameters measures and ultrastructure. The characteristics of the cryopreserved cells were compared with those of non-cryopreserved cells. Additionally, cryopreserved cells were evaluated for viability immediately after thawing and in vitro culture.

2.8 | Evaluations of cell quality

The cell morphology was analyzed daily using an inverted microscope (da Silva Viana et al., 2023) for the observation of cytoplasmic extensions, colony formation, cell detachment, and confluence. For the viability assay, cells were stained with trypan blue solution (0.4% in PBS) at 1:1 ratio (Rodrigues et al., 2023). Cell numbers were conducted using a Neubauer chamber, where stained cells with ruptured membranes were quantified as nonviable, while unstained cells with intact membranes were considered viable. The viability was determined by viable cells/total cell counted × 100.

Apoptosis levels were determined using acridine orange (2.0 µg/mL) and ethidium bromide (10 µg/mL, Lira et al., 2022). After incubation, cells were examined under a fluorescence microscope, and the acquired images were subjected to analysis using Image J software (National Institutes of Health, NIH, USA) to quantify the fluorescence signal intensity. Cells were classified based on their characteristics: those with a light green nucleus were deemed viable. Cells exhibiting nonuniform aspects were categorized as early apoptosis. Cells displaying bright orange areas were identified as being in late apoptosis. Lastly, cells with a uniformly orange nucleus were classified as necrotic.

Metabolic activity was analyzed through the conversion of (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into formazan crystals. A cell density of 5.0×10^4 cells/mL was cultured in 12-well dishes for 5 days under conditions of 38.5°C and 6.5% CO₂. After incubation, cells were treated with MTT solution (5 mg/mL in DMEM) for 3 h (Silva et al., 2021). Subsequently, formazan crystals were solubilized using DMSO and the absorbance was measured using a spectrophotometer (Shimadzu® UVmini-1240) at a wavelength of 595 nm.

The population doubling capacity analysis and growth curve determination were carried out following the methodology described by Rodrigues et al. (2023). A concentration of 7.0×10^4 cells/mL was evenly distributed across seven wells in a 24-well dish. Each well was trypsinized and quantified every 24 h over a total period of 168 h. The cell concentrations measured at each 24-h interval were utilized to construct the growth curve and calculate the population doubling time (PDT) using the formula: $PDT = (T \times \ln 2) / \ln (X_e / X_b)$, where T represents the incubation time, X_b and X_e, respectively, denote the initial and final cell counts at the beginning and end of the incubation period, and ln refers to the natural logarithm.

Fluorescence probes, 5 µM 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) and 500 nM MitoTracker Red (CMXRos) were used to assess ROS levels and ΔΨ_m. Cell suspensions (100 µL each) were exposed to the respective probe for 30 min under standard cell culture conditions (38.5°C and 6.5% CO₂). Subsequently, cells underwent centrifugation (300 × g for 3 min), followed by visualization using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). Fluorescence intensity was quantified using Image J software (National Institutes of Health, NIH, USA).

Finally, non-cryopreserved and cryopreserved cells were assessed for ultrastructural damage, focusing on alterations to the cell surface and cytoplasmic extensions. For evaluation, cells were fixed in 4% glutaraldehyde for 12 h, followed by contrast fixation with 1% osmium tetroxide for 1 h, and dehydration through a graded alcohol series (Lira et al., 2022). Cells were metalized and subsequently visualized using a scanning electron microscope (TESCAN VEGA3; Tescan Analytics, Fuveau, Bouches-du-Rhône, France).

2.9 | Statistical analysis

A total of four cell lines were used, being one cell line per animal. All the cell lines were submitted to the analysis. Data were expressed as mean ± standard error, considering one animal/one cell line/one repetition, and analyzed using GraphPad software (Graph-Pad Software Incorporation, La Jolla, USA). Normality and homoscedasticity were performed in all results using Shapiro-wilk test and Levene's test, respectively. All data were altered with arcsine and analyzed using analysis of variance (ANOVA) followed by the Tukey test or unpaired t-test. Statistical significance was considered when $p < .05$.

3 | RESULTS

3.1 | Cell type confirmation and karyotype analysis

Cell isolation was conducted using 4.3 ± 0.3 explants per animal. Notably, each animal exhibited variability in both the quantification of explant attachment and the subconfluence (Table 1). This variability was further evident across days of attachment and subconfluence. Moreover, this variability persisted throughout subsequent different passages, with cells reaching 70% confluence at the third, seventh, and eleventh passages after 26.5 ± 0.3 , 43 ± 0.6 and 96.7 ± 8.3 days, respectively.

The cell type was confirmed through vimentin identification using immunofluorescence assay, where cytoplasmic extensions exhibited strong green fluorescence (Figure 1a), while the nucleus was delineated by blue fluorescence emission (Figure 1b). The merged images facilitated the comprehensive visualization of both the

TABLE 1 Description of primary cell culture of somatic tissues and cells derived from northern tiger cat.

Animal (Sex)	No. samples			Days		
	Initial	Attached (%)	Subconfluence (%)	Attached	Subconfluence	Total cell culture
A1 (M)	5	100 (5/5)	100 (5/5)	4	9	15
A2 (M)	4	75 (3/4)	75 (3/4)	1	8	16
A3 (M)	4	100 (4/4)	100 (4/4)	1	8	17
A4 (F)	4	100 (4/4)	75 (3/4)	3	10	17
Mean ± S.E.	4.3 ± 0.3	94.0 ± 6.0	88.0 ± 7.0	2.3 ± 0.8	8.8 ± 0.5	16.5 ± 0.5

Note: M, Male; F, Female; Mean ± Standard Error. $p > .05$.

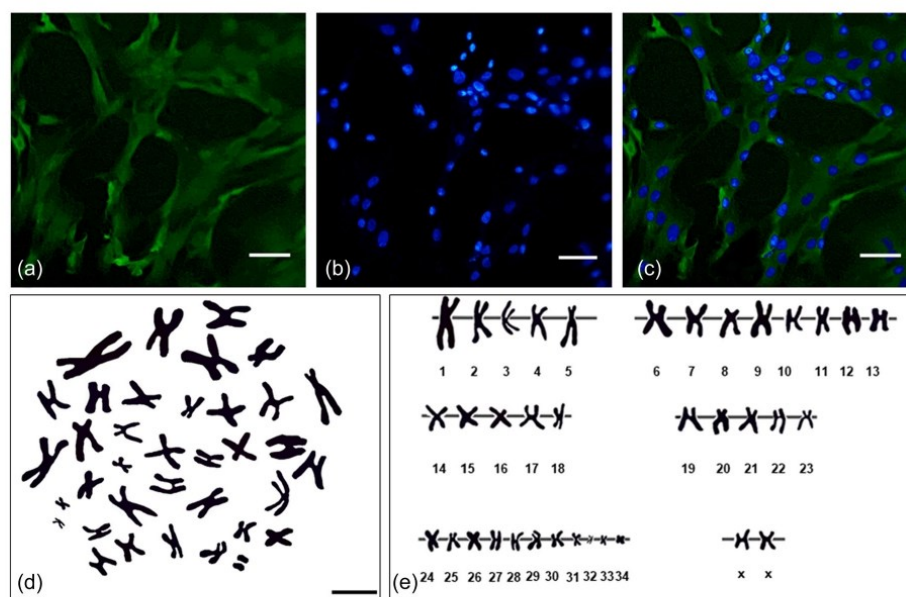


FIGURE 1 Cell type characterization using immunophenotyping and karyotyping assays in northern tiger cat. (a) Fibroblasts of one animal (A2, male) stained with vimentin (green) identifying the cytoplasmic projection. (b) The same fibroblasts of animal stained with Hoechst 33342 (blue) identifying the nucleus. Scale bar 50 μ m, 40 $\times$. (c) Overlapping stained nucleus and vimentin. (d, e) Karyotype of one animal (A2, male) in the third passage, $n = 36$. Scale bar 100 μ m, 100 $\times$.

nucleus and the cytoplasmic projections as fibroblasts (Figure 1c). Karyotype analysis revealed a diploid count of $2n = 36$ chromosomes (Figure 1d), with a fundamental number (FN) of 68. This analysis enabled the identification of 34 autosomal chromosomes and two sex chromosomes in male (Figure 1e), classified as 26 metacentric chromosomes and eight submetacentric chromosomes.

Moreover, cells were cultured without the presence of antibiotic/antimycotic agents, and throughout the 27-day period, no changes in color, viscosity, or turbidity of the medium were observed. Additionally, no significant alterations in cell proliferation were observed. Importantly, no evidence of fungal structures or bacterial contamination was detected during this period.

3.2 | Effects of extended passages on culture quality

The primary culture exhibited typical heterogeneity, containing fibroblasts and circular cells (Figure 2a). Fibroblasts, characterized by oval and central nuclei, along with fusiform morphology and numerous cytoplasmic projections, were observed at the third (Figure 2b) and seventh (Figure 2c) passages. However, morphological alterations, including increased cell size and decreased cytoplasmic projections, were evident at the eleventh passage (Figure 2d). Even with these changes, viability rates remained stable during the

passages (Figure 3a), with no differences observed between the third ($91.7\% \pm 4.2$), seventh ($84.5\% \pm 5.8$), and eleventh ($86.2\% \pm 5.9$) passages ($p > .05$).

Contrasting with the viability, metabolic activity decreased progressively from the third ($91.2\% \pm 5.2$) to the seventh ($56.0\% \pm 2.9$) and eleventh ($22.8\% \pm 3.8$) passages (Figure 3b). Interestingly, the apoptotic levels remained unchanged between the passages (Figure 3c, c' and C'), resulting in a high percentage of viable cells observed at the third ($98.7\% \pm 0.4$), seventh ($91.4\% \pm 3.3$), and eleventh ($74.5\% \pm 13.6$) passages, and no increases were noted in early apoptotic, late apoptotic, or necrotic cells (Figure 3d).

Additionally, PDT remained unaffected between the third ($42.5 \text{ h} \pm 4.0$) and seventh ($39.8 \text{ h} \pm 8.3$) passages (Figure 3e). However, at the eleventh passage, only one animal exhibited an adequate cell concentration and was analyzed, revealing an elevated PDT of 85.4 h. The growth curve displayed a sigmoidal shape at both the third and the seventh passages (Figure 3f), characterized by similar exponential phases lasting around 2 days, followed by stationery and latency phases. Interestingly, at the eleventh passage, the exponential phase occurred later and was succeeded by a decrease in cell concentration in the subsequent days.

Despite the prolonged cell culture duration, ROS levels remained consistent between the third (Figure 4a), seventh (Figure 4b), and eleventh (Figure 4c) passages (1.00 ± 0.73 arbitrary fluorescence units (AFU) vs. 0.87 ± 0.58 AFU vs. 0.92 ± 0.63 AFU, respectively) (Figure 4d).

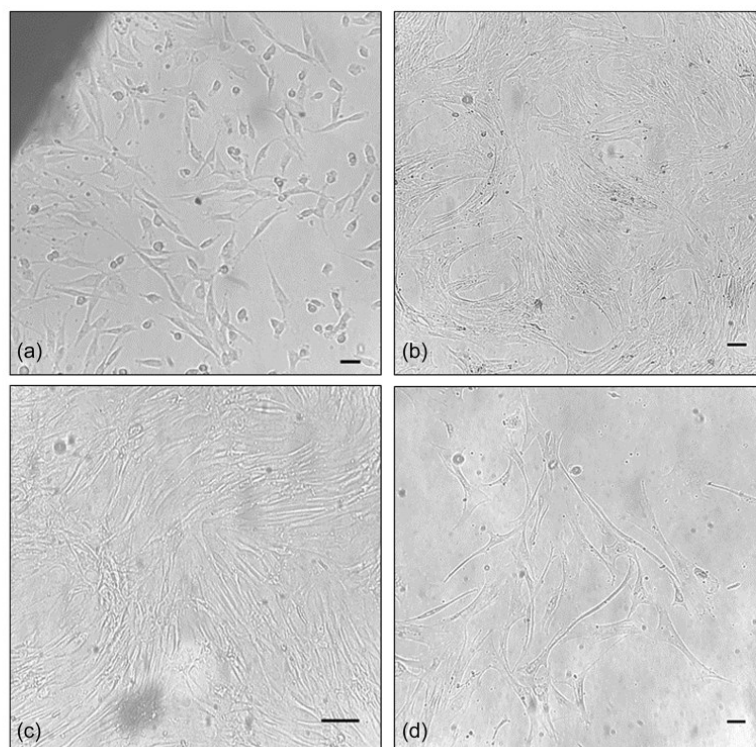


FIGURE 2 Evaluation of morphology of fibroblasts derived from northern tiger cat after extended cell culture. Fibroblasts during (a) primary cell culture, (b) third, (c) seventh, (d) eleventh passage. Scale bar 50 μ m, 20 $\times$.

Assessments indicated an increase in $\Delta\Psi_m$ (Figure 4e, f and g) at the seventh (2.33 ± 1.35 AFU) and eleventh (2.35 ± 1.14 AFU) passages compared to the third passage (1.00 ± 0.62 AFU) (Figure 4h).

3.3 | Effects of cell cryopreservation on culture quality

Morphological changes were not observed under light microscopy (Figures 5a and 5b), with cells maintaining their fusiform shape, central nucleus, and numerous cytoplasmic projections. However, ultrastructural analysis revealed significant differences between the non-cryopreserved and cryopreserved cells. Non-cryopreserved cells exhibited large size and extensive cytoplasmic projections (Figure 5a), whereas the cryopreservation led to a reduction in cytoplasmic projections (Figure 5b).

Initial post-thawing viability was notably impacted ($64.7\% \pm 3.9$), but in vitro culture increased the viability ($95.6\% \pm 1.5$), approaching levels observed in non-cryopreserved cells ($91.7\% \pm 4.2$, Figure 6a). Furthermore, metabolic activity (Figure 6b) was preserved after cryopreservation ($91.2\% \pm 5.2$ vs. $72.7\% \pm 6.5$). Apoptotic levels were unchanged, with viable cells (Figure 6c) comprising $98.7\% \pm 0.4$ pre-cryopreservation and

$95.1\% \pm 2.9$ post-cryopreservation, contributing to low rates of early apoptotic (Figure 6d), late apoptotic (Figure 6e), and necrotic cells (Figure 6f), all around 0.3% (Figure 6g).

Parameters of cell division such as PDT (Figure 6h) remained unaffected by cryopreservation (non-cryopreserved cells: $42.5 \text{ h} \pm 4.0$ vs cryopreserved cells: $47.1 \text{ h} \pm 9.9$), and the cell growth curve (Figure 6i) exhibited similar sigmoidal shapes with defined exponential, latency, and stationary phases. Additionally, cryopreservation led to a decrease in ROS levels, how it is observed in the fluorescence before (Figure 7a) and after cryopreservation (Figure 7a'), reducing from 1.00 ± 0.73 to 0.75 ± 0.58 AFU (Figure 7b). The stress induced by cryopreservation was evident in $\Delta\Psi_m$ after cryopreservation (Figure 7c vs. Figure 7c'), which increased from 1.00 ± 0.62 AFU to 3.79 ± 1.61 AFU after cryopreservation (Figure 7d).

4 | DISCUSSION

Arantes et al. (2021) were pioneered in the isolation, ultrastructure characterization, and cryopreservation of somatic cells derived from northern tiger cat. Differently, our study contributes to validate the

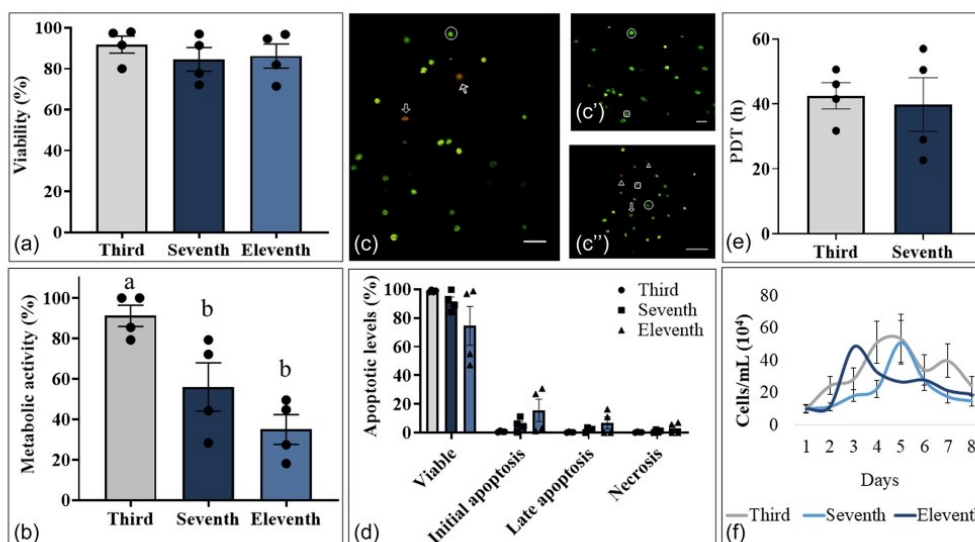


FIGURE 3 Viability and proliferation of cells derived from northern tiger cat cell after extended cell culture (third, seventh, and eleventh passages). (a) Cell viability by trypan blue assay. $p > .05$. (b) Metabolic activity determined by the MTT assay. (a, b): Values with different superscript represent significant differences ($p < .05$). Apoptosis levels by identification with acridine orange and ethidium bromide after (c) third, viable cells (Circle); Necrotic cells (Arrow), (c') seventh, viable cells (Circle); Initial apoptotic (Square), and (c'') eleventh passage, viable cells (Circle); Initial apoptotic (Square); Later apoptotic (Triangle); Necrotic cells (Arrow). (d) Apoptotic levels quantification. $p > .05$. (e) Population double time (PDT) after seven days of cell culture. $p > .05$. (f) Growth curve after seven days of cell culture. $p > .05$. Scale bars 50 μ m, 40 $\times$ (c, c' and c'').

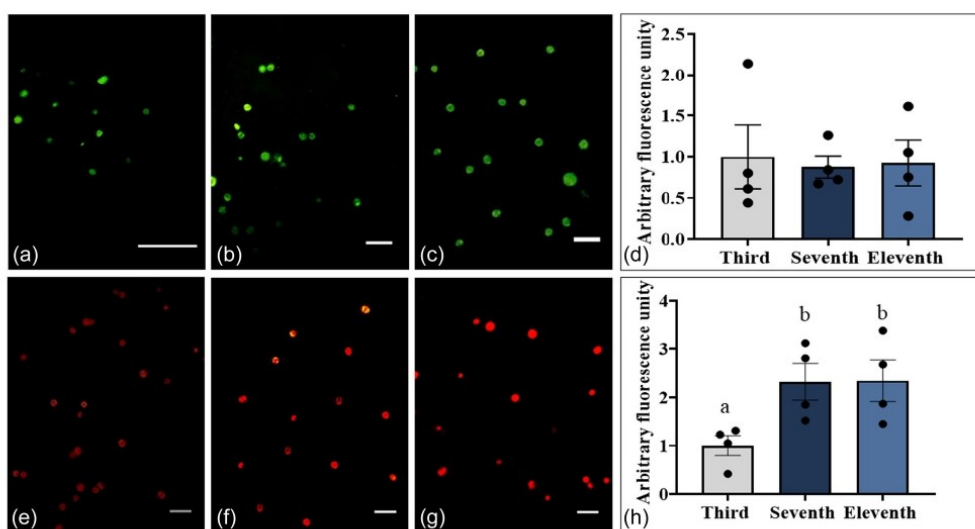


FIGURE 4 Oxidative stress and potential mitochondrial membrane after third, seventh, and eleventh passages in cells derived from northern tiger cat. Marked cells with H_2DCFDA during (a) third, (b) seventh and (c) eleventh passage. (d) ROS level quantification in arbitrary fluorescence units. Image of potential mitochondrial membrane marked with MitoTracker Red during (e) third, (f) seventh and (g) eleventh passages. (h) Potential mitochondrial membrane using arbitrary fluorescence units measurement; Scale bars 50 μ m, 40 $\times$; (a, b): Values with different superscript represent significant differences ($p < .05$).

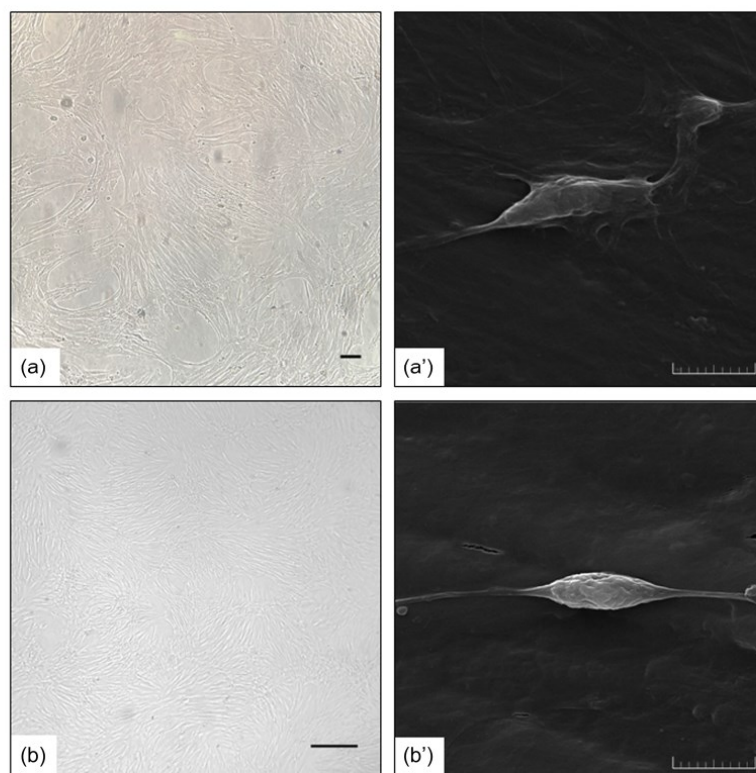


FIGURE 5 Morphology and ultrastructure of fibroblasts after cryopreservation derived from northern tiger cat. Morphology of (a) non-cryopreserved and (b) cryopreserved fibroblasts under an inverted microscope. Scale bar: 50 μm , 20 $\times$. (a') Ultrastructure of non-cryopreserved and (b') cryopreserved cells with a fusiform shape and cell projections. Scale bar: 5 μm .

cell type by immune identification and offers karyotype characterization for the establishment of stable cell lines. Additionally, recognizing the potential stress induced by cell culture and cryopreservation, the study investigated the effects of extended cell passages and cryopreservation.

The confirmation of purity of cell culture was performed at third passage, with vimentin, a filament founded in fibroblasts cytoskeleton. The expression of vimentin makes the cell identification possible and can be an indicative of cell quality biophysics (Sliogeryte & Gavara, 2019). Moreover, the analyzed complete karyotype is an important key for genetic studies and conservation of species. In wild felids, for example, the right accessibility of genetic material has been used to study the evolution of tigers and demographic history (Khan, 2022). Moreover, karyotype analysis had been an essential tool for somatic cell nuclear transfer (SCNT), trying to avoid the number of abnormalities in produced embryos (Song et al., 2007; Veraguas et al., 2020).

The time for subconfluence during the primary culture of northern tiger cat cells was similar, as described for other wild felids,

such as ocelot (*Leopardus pardalis*, da Silva Viana et al., 2023) and puma (*P. concolor*, Lira et al., 2022), with a mean value of 16 days. Additionally, the primary culture showed a heterogeneity of cells with shapes similar to fibroblasts and keratinocytes. Also, the homogeneity of fibroblasts after three subcultures was similar to the cell line of fishing cat (*Prionailurus viverrinus*, Sukparangsi et al., 2022).

The growth curve of northern tiger cat was described by Arantes et al. (2021) with an increase in cell concentration after 24 h. However, these authors did not calculate PDT precisely. Differently, in our study, PDT was around 40 h, similar to other felids, such as tiger (*Panthera tigris tigris*), with PDT at 34.7 h (Yelliseti et al., 2016), and puma, with PDT at 31 h (Lira et al., 2022). Additionally, cells of northern tiger cat decreased PDT and changed the growth curve during the eleventh passage, similar to puma (Lira et al., 2022) and jaguar (Silva et al., 2021). Madelaire et al. (2022) indicated a species-specific resistance of cell passages, due to the physiology and natural habits. Apparently, wild felid cells have a sensitivity to in vitro stress after the tenth passage, diverging of other species, such as Chinese muntjac (*Muntiacus reevesi*), at the eleventh passage (Wang

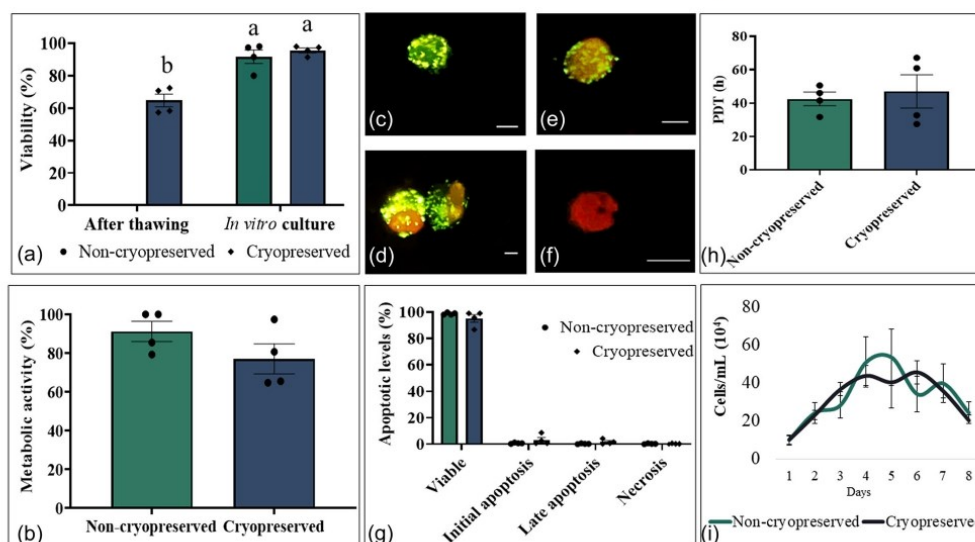


FIGURE 6 Evaluation of cell parameters of northern tiger cat cells after cryopreservation. (a) Viability measured with trypan blue. (b) Metabolic activity measurement with MTT assay. Cells of third passage marked with acridine orange and ethidium bromide (c) Viable cells, (d) initial apoptotic, (e) late apoptotic (f) necrotic cells. (g) Quantification of apoptosis levels. (h) Growth curve. (i) Population double time; (c, d, e, and f) Scale bars 50 μ m, 100 $\times$, $p > .05$.

et al., 2020), or hippopotamus (*Hippopotamus amphibius*), at the twelfth passage (Wang et al., 2021).

The effect of senescence markers was also observed on mitochondrial activity between the seventh and eleventh passages. In general, the senescent cells had a decreased metabolic activity occasioned by DNA damage and lower quantification of NAD^+/NADH (Shmulevich & Krizhanovsky, 2021). Even with damages, cell viability and apoptotic levels were unaltered. Probably, these cells could be in a replicate senescence, where the limitation of in vitro cell duplication happens (Kamal et al., 2020).

The damage occasioned to mitochondria was also observed in the increase in $\Delta\Psi_m$ and ROS levels. The increase in $\Delta\Psi_m$ can be explained by the increase in mitochondria mass, and these modifications can decrease the ROS levels by the increase in electron transport (Miwa et al., 2022). Additionally, the necessity of repairing the cell damage during extended cell culture results in an ATPs necessity, increasing the $\Delta\Psi_m$ levels (Zorova et al., 2018).

Understanding these effects of cell passage on northern tiger cat cells can contribute to the next steps for conservation of species. For wild mammals that can be affected by the prolonged cell culture, the medium supplementation could be optimized, such as in fishing cat (Sukparangsi et al., 2022), or African savanna elephants (*Loxodonta africana*) (Van Vuuren et al., 2023). This information can contribute specially for efficient reprogramming in induced pluripotency stem cells (iPSC, Sukparangsi et al., 2022). Also, the cell line can be applied as an important physiology study tool, aiding to understand

differences between species and their adaptability (Madaire et al., 2022; Pickering et al., 2015).

Moreover, the cryotolerance was an important parameter for the establishment of cell lines, as observed in other species, such as Siberian tiger (*Panthera tigris altaica*, Liu et al., 2010), Iberian lynx (*Lynx pardinus*, León-Quinto et al., 2011), and Bengal tiger (*Panthera tigris tigris*, Guan et al., 2010). Arantes et al. (2021) performed the cryopreservation of somatic cells of northern tiger cat with 2.5% DMSO, with efficiency in the viability and lower number of necrosis cells, which was similar to our study. Additionally, these results were achieved by DMSO being effective in the membrane permeability at low temperature, which can facilitate the cell dehydration during cryopreservation, recovering cells with higher viability (Jungare et al., 2022). In our study, 2.5% DMSO were effective in the proliferative and metabolic activity. However, 2.5% DMSO affected the $\Delta\Psi_m$ and ROS levels of cells of the northern tiger cat. Lower ROS levels can affect the cell equilibrium, and in normal levels these molecules were associated with interaction with phosphatases, kinases, and cell proliferation (Brillo et al., 2021).

Recently, lower exposure concentration of DMSO has been applied due to the less toxicity in metabolic activity, enzymes functions, and apoptosis (Erol et al., 2021). On the other hand, in puma cell cryopreservation, the increase in DMSO concentration with 0.2 M sucrose promoted the conservation of the ROS levels. The use of DMSO with extracellular cryoprotectants has showed greater results, once DMSO increases the membrane permeability

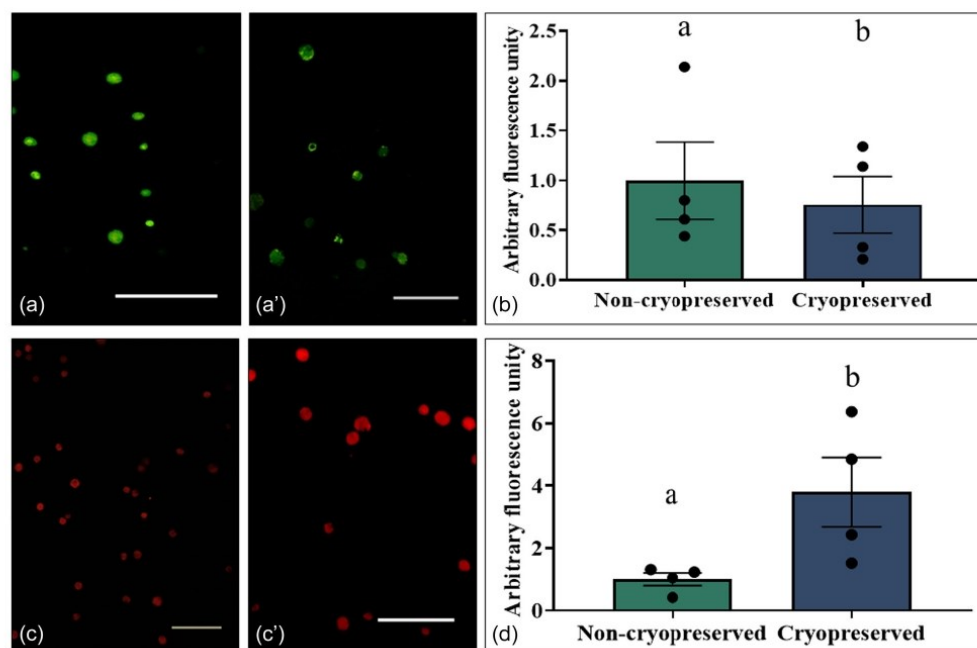


FIGURE 7 Evaluation of ROS levels by H₂DCFDA and mitochondrial membrane potential with MitoTracker Red after northern tiger cat cells cryopreservation. (a) Non-cryopreserved and (a') cryopreserved cells marked with H₂DCFDA. (b) Measurement of arbitrary fluorescence units for ROS levels quantification; (c) Non-cryopreserved and (c') cryopreserved cells marked with MitoTracker Red. (d) mitochondrial membrane potential evaluation by arbitrary fluorescence unity quantification. Scale bars 50 μm, 40x. (a, b): Values with different superscript represent significant differences ($p < .05$).

in extracellular cryoprotectants (Whaley et al., 2021). Probably, adjustments in the use of extracellular cryoprotectants in the small wild cat cell cryopreservation solution could improve conservation efficiency.

In conclusion, fibroblasts from northern tiger cat were affected by extended passage (seventh and eleventh passages) and cryopreservation. Adjustments to the in vitro culture and cryopreservation are necessary to reduce cellular oxidative stress caused by in vitro conditions. These results described, at first, the cellular characteristics according to different quality parameters of northern tiger cat samples during extended in vitro culture and cryopreservation, providing a valuable source for assisted reproduction techniques, aiming at the genetic conservation of this species.

AUTHOR CONTRIBUTIONS

J.V.S. Viana (Investigation, Visualization, Methodology, Writing), L.R.M. Oliveira, L.L.V. Rodrigues, Y.L.F. Silva, A.L.R. Rodrigues (Investigation, Visualization, Methodology); A.R. Silva, P.V. Alves, H.V.R. Silva (Conceptualization, Methodology); A.F. Pereira (Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions. The data that support the findings of this study are available on request from the corresponding author (A.F. Pereira).

ORCID

Alexandre Rodrigues Silva <http://orcid.org/0000-0001-6027-1062>

Alexandra Fernandes Pereira <http://orcid.org/0000-0003-2137-854X>

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3043 ANEXO No. 2: ARTIGO “NO SYNERGISTIC EFFECT OF EXTRACELLULAR
3044 CRYOPROTECTANTS WITH DIMETHYL SULFOXIDE IN THE CONSERVATION OF
3045 NORTHERN TIGER CAT FIBROBLASTS” PUBLICADO

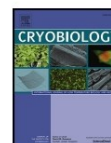
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No synergistic effect of extracellular cryoprotectants with dimethyl sulfoxide in the conservation of northern tiger cat fibroblasts

João Vitor da Silva Viana^a, Lhara Ricarliany Medeiros de Oliveira^a,
Luanna Lorena Vieira Rodrigues^a, Yasmin Beatriz França Moura^a,
Antonia Beatriz Mendonça Pereira^a, Patrícia Vasconcelos Alves^b,
Herlon Victor Rodrigues Silva^c, Alessandra Fernandes Pereira^{a,*}

^a Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoró, RN, Brazil

^b Sargento Prata Municipal Zoo, Fortaleza, CE, Brazil

^c Laboratory of Carnivores Reproduction, University of Ceará State, Fortaleza, CE, Brazil

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ABSTRACT

The success of somatic cell cryobanks is dependent on establishing reproducible cryopreservation methodologies. We supposed that associated extracellular cryoprotectants (sucrose and L-proline) with 2.5 or 10 % dimethyl sulfoxide (Me₂SO) could guarantee better northern tiger cat cells quality rates after thawing when compared to Me₂SO alone. Therefore, we evaluated the effects of sucrose or L-proline with 2.5 or 10 % Me₂SO on the cryopreservation of northern tiger cat fibroblasts. Somatic cells were also cryopreserved with 2.5 % or 10 % Me₂SO alone. All cells were analyzed for morphology, membrane integrity, proliferative activity, metabolism, apoptosis classification, reactive oxygen species (ROS) levels, and mitochondrial membrane potential ($\Delta\Psi_m$). Regardless of the cryoprotective solution, cryopreservation did not affect morphology, membrane integrity after culture, proliferative activity, and metabolism ($P > 0.05$). However, immediately after thawing, 2.5 % Me₂SO with L-proline and 10 % Me₂SO promoted higher rates of membrane integrity when compared to the other cryopreserved groups ($P < 0.05$). Interestingly, cells cryopreserved with 10 % Me₂SO maintained ROS levels similar to non-cryopreserved cells ($P > 0.05$). However, the percentage of viable cells evaluated by apoptosis classification was reduced when using 10 % Me₂SO with L-proline compared to non-cryopreserved groups ($P < 0.05$). Additionally, $\Delta\Psi_m$ was altered in all cryopreserved groups ($P < 0.05$). In summary, sucrose and L-proline were less effective in cryopreservation of northern tiger cat fibroblasts in the presence of 2.5 % or 10 % Me₂SO. Also, 10 % Me₂SO appears to be the most suitable cryoprotectant for the formation of cryobanks of this species.

1. Introduction

The northern tiger cat (*Leopardus tigrinus*, Schreber, 1775) is a small wild felid from Brazil with ecological impact on ecosystem balance [10]. According to the International Union for Conservation of Nature (IUCN) Red List, they are categorized as vulnerable to extinction due to human interference [24]. Given this scenario, conservation strategies have been proposed, such as somatic cell cryobanks [1]. These cryobanks are important tools for the conservation of wild felids [20,25] and have been created in different regions mainly due to the global concern for maintaining biodiversity [14,30].

Arantes et al. [1] developed the first study in northern tiger cats for

somatic cell cryobanks. The authors observed that 2.5 % and 10 % dimethyl sulfoxide (Me₂SO) efficiently protected the somatic cells of the northern tiger cats. Although reduced concentrations of Me₂SO emerge as new options for the cryopreservation of somatic cells of wild felids [1, 27], by reducing intracellular cryoprotectant toxicity, we hypothesized that this toxicity could also be reduced with the addition of extracellular cryoprotectants [16,17]. These extracellular cryoprotectants can prevent apoptosis and osmolarity damage during cryopreservation [3]. Additionally, we observed that systematic studies to evaluate different cryoprotectants in somatic cell preservation in northern tiger cats have been limited.

A new generation of extracellular cryoprotectants has been

* Corresponding author. Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil.
E-mail address: alexandra.pereira@ufersa.edu.br (A.F. Pereira).

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discovered based on its natural origin [26]. L-proline is an extracellular cryoprotectant that can reduce the formation of large ice crystals [19]. Interestingly, L-proline showed synergism with Me₂SO, increasing the cell viability and reducing the reactive oxygen species (ROS) levels, especially at lower concentrations of Me₂SO (5 % or 7.5 %) in human epithelial cells [8]. The effects of L-proline on ice crystal were described at concentrations >100 mM, influencing cell metabolism [3].

Sucrose is also an extracellular cryoprotectant, acting on membrane stability and osmotic cell regulation, and its synergism improves Me₂SO action [7]. In somatic cells of wild felids, 0.2 M sucrose with 10 % Me₂SO increased metabolic activity in the Iberian lynx (*Lynx pardinus*) from 50 to 80 % [16]. This combination in jaguar (*Panthera onca*) somatic cells conserved the viability and metabolic activity [22]. However, in pumas (*Puma concolor*), sucrose did not increase the viability or metabolic activity of somatic cells [28].

Therefore, since the success of somatic cell banks depends on optimizing the choice of cryoprotectants and on reducing the toxicity of intracellular cryoprotectants, we evaluated the synergic effects of sucrose and L-proline with 2.5 or 10 % Me₂SO on the cryopreservation of northern tiger cat fibroblasts.

2. Material and methods

2.1. Chemicals and media

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin solution, and fluorescence probes were obtained from Gibco-BRL (Carlsbad, CA, USA). All the other chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Ethics statement and ear skin collection

The experimental protocols were approved by the Animal Ethics Committee (CEUA/UFERSA, no. 20/2023) and the Chico Mendes Institute for Biodiversity Conservation (no. 88201-1; ICMBio). Ear skin samples (1.0–1.5 cm²) of three males and one female derived from zoos in Ceará, with ages between 2 and 3 years, were used. All samples were transported at 4 °C for 4–5 h to the laboratory in DMEM with 10 % FBS and 2 % penicillin and streptomycin solution.

2.3. Cell isolation

The ear skin of each animal was fragmented into 6.0 mm³ (3 × 2 × 1 mm) and cultured in DMEM with 10 % FBS, 2 % penicillin, streptomycin solution at 38.5 °C, and 6.5 % CO₂. Fragments were cultured until the somatic cells surrounded the tissues. Subsequently, cells were cultured until 70 % confluence and submitted to the first passage. Cells were cryopreserved using different cryopreservation solutions after the third passage, being four days for each passage, and after characterization as fibroblasts [12].

2.4. Experimental design

Fibroblasts were cryopreserved in six solutions containing DMEM, 10 % FBS, and (i) 2.5 % Me₂SO, (ii) 2.5 % Me₂SO with 0.2 M sucrose, (iii) 2.5 % Me₂SO with 100 mM L-proline, (iv) 10 % Me₂SO, (v) 10 % Me₂SO with 0.2 M sucrose, and (vi) 10 % Me₂SO with 100 mM L-proline. Cells not subjected to cryopreservation were used as controls. All cells were analyzed for morphology, membrane integrity, proliferative activity, metabolism, apoptosis classification, ROS levels, and mitochondrial membrane potential (ΔΨm).

2.5. Fibroblast cryopreservation by slow freezing

The cryopreservation was performed as described by Rodrigues et al. [28]. A 1.0 × 10⁵ cell/mL concentration was allocated in cryovials and

exposed to the cryopreservation solution only with Me₂SO for 15 min at 4 °C. For the solution with sucrose or L-proline, cells were exposed for an additional 15 min at 4 °C. All cryovials were transferred to a freezing container (Mr. Frosty, Thermo Scientific Nalgene, Rochester, NY, USA), and the container was placed in a –80 °C freezer (1 °C/min) cooling rate until –80 °C. Then the cryovials were plunged into liquid nitrogen (–196 °C) for storage.

After two weeks, all the groups were thawed at 25 °C (1 min) and 37 °C (4 min). The groups with sucrose or L-proline were diluted in a solution with 0.2 M sucrose or 100 mM L-proline and maintained at 4 °C for 15 min [28]. Subsequently, the cells were centrifuged for 10 min at 600×g and resuspended in DMEM. After the extracellular cryoprotectant removal, all the groups were kept for 15 min at 25 °C followed by another centrifugation. Finally, 10 µL cell suspension was evaluated for membrane integrity, and the remaining cells were cultured until 70 % confluence and subject to the other analyses described below.

2.6. Evaluation of cell morphology

The cells under an inverted microscope (Leipzig IMx 400, Phoenix-Optics, Germany) were evaluated every 48 h for morphology characteristics, such as cytoplasmic projections, nucleus, and the presence of granules or vacuoles [1]. Additionally, medium color changes, the presence of fungal or bacterial contamination, and time until the subconfluence were observed.

2.7. Evaluation of cell membrane integrity, proliferative activity, and metabolism

Cell membrane integrity was evaluated by the cell's permeability to 0.4 % trypan blue (in phosphate-buffered saline, PBS) by mixing 1:1 of cell suspension and trypan blue [22]. The viability was evaluated immediately after thawing and after one week of *in vitro* cell culture. Cell numbers were conducted using a Neubauer chamber. Dead cells were permeable to the stain and identified as blue, and viable cells with intact membranes were identified by the absence of color. The viability was determined by cells with intact membrane/total cell counted × 100.

The proliferative capacity was measured by population double time (PDT) in hours [18]. A 7.0 × 10⁴ cells/mL concentration was initially distributed into 7 wells of 24-well dishes. Each dish was counted in intervals of 24 h for up to 168 h. Daily, cells were trypsinized (0.25 % trypsin-EDTA solution) and quantified using a Neubauer chamber, and concentrations were used to elaborate the growth curve. The PDT was measured according to the formula: $PDT = T \ln 2 / \ln (X_e/X_b)$, where T is the incubation time, X_b is the number of cells at the beginning of the incubation period, X_e is the number of cells at the end of the incubation period, and ln is the natural logarithm.

The metabolism was determined by the cell's ability to metabolize 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT assay) in formazan crystals [28]. Initially, a 5.0 × 10⁵ cell/mL concentration was cultured for five days in the same culture conditions, and then incubated in MTT solution (5 mg/mL in DMEM) for 3 h at 38.5 °C and 6.5 % CO₂. After the metabolization, formazan crystals were diluted with 1.0 mL Me₂SO, and the absorbance was measured at 595 nm (Shimadzu® UVmini-1240, Kyoto, Japan).

2.8. Evaluation of apoptosis classification

The apoptotic classification was measured by fluorescence, where uniform green, non-uniform green, orange, and red were considered viable, initial apoptosis, later apoptosis, and necrotic cells, respectively [29]. Briefly, 50 µL cell suspension was exposed to a 2.0 µg/mL acridine orange solution and 10 µg/mL ethidium bromide for 4 min and images were captured using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at 40 × magnification. For each group, 300 cells were counted and classified using the cell counter of Image J software

(National Institutes of Health, NIH, USA).

2.9. Evaluation of reactive oxygen species (ROS) levels and mitochondrial membrane potential ($\Delta\Psi_m$)

ROS levels and $\Delta\Psi_m$ were evaluated using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) and MitoTracker Red (CMXRos) probes, respectively. For each probe, cells and probes were mixed (1:1) and incubated for 30 min at 38.5 °C and 6.5 % CO₂ [18]. Subsequently, stained cells were photographed under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan) at 40 × magnification. Photomicrographs were evaluated for fluorescence intensity (pixels) using Image J software. Each cell area ~150 μm² was used to measure the fluorescence intensity. The background fluorescence was measured per cell. The final value was the result of the: cell fluorescence = initial cell fluorescence - background fluorescence.

2.10. Statistical analysis

All the data were expressed as mean ± standard error (one animal/repetition), and analysis was performed using GraphPad software (Graph-Pad Software Incorporation, La Jolla, CA). The Shapiro-Wilk and Levene tests verified the normality and homoscedasticity, respectively. Data was not normally distributed hence arcsine transformed and analyzed by ANOVA followed by the Tukey test. Significance was set at $P < 0.05$.

3. Results

3.1. Evaluation of cell morphology

Before cryopreservation, cells showed fibroblast morphology with a fusiform shape, central and oval nucleus, cytoplasmic projection, and translucent cytoplasm (Fig. 1A). After cryopreservation (Fig. 1B–G), all the solutions conserved the cell morphology, and the time for 70 % confluence was approximately 15 days for all the groups.

3.2. Evaluation of cell membrane integrity, proliferative activity, and metabolism

Immediately after thawing, 2.5 % Me₂SO with L-proline (80.0 % ±

1.8) and 10 % Me₂SO (79.4 % ± 6.1) promoted higher rates of membrane integrity when compared to the other cryopreserved groups with values between 61 % and 69 % (Fig. 2A). However, regardless of the cryoprotective solution, the membrane integrity after *in vitro* culture were kept over than 90 %.

Moreover, no difference was observed between non-cryopreserved and cryopreserved cells for PDT (Fig. 2B). All PDT showed values ranging from 30 h to 70 h ($P > 0.05$). Moreover, 10 % Me₂SO and 10 % Me₂SO-sucrose had a slightly higher PDT of 70 h when compared to 2.5 % Me₂SO-sucrose and 2.5 % Me₂SO-L-proline around 30 h. Additionally, all the groups showed growth curve similarities, with defined latency, exponential, stationary, and plateau phases (Fig. 2C).

Regardless of the cryoprotective solution, cryopreservation did not affect metabolism ($P > 0.05$), ranging from 60 % to 100 % (Fig. 2D). In particular, the group 2.5 % Me₂SO-sucrose (100.0 % ± 1.8) had slightly higher metabolism than the groups 10 % Me₂SO (63.8 % ± 7.9), 10 % Me₂SO-sucrose (63.3 % ± 8.7) and 10 % Me₂SO-L-proline (60.2 % ± 10.3).

3.3. Evaluation of apoptosis classification

The different levels of apoptotic cells were quantified for all groups (Fig. 3A–G). The 10 % Me₂SO-L-proline solution decreased ($P < 0.05$) the viable cells (81.3 % ± 9.6) and increased the number of late apoptotic cells (8.3 % ± 5.5, Fig. 3H) compared to non-cryopreserved cells. All the other solutions maintained the viable cell number similar ($P > 0.05$) to non-cryopreserved cells (98.7 % ± 0.4, Fig. 3H). Additionally, these solutions showed a lower number of initial apoptotic (~2 %), late apoptotic (~1 %), and necrotic cells (~1 %).

3.4. ROS levels and $\Delta\Psi_m$

The solution with 10 % Me₂SO did not affect ($P > 0.05$) the ROS levels when compared to non-cryopreserved cells (Fig. 4A–B). Moreover, the other solutions decreased or increased ($P < 0.05$) ROS levels when compared to non-cryopreserved cells.

All the groups increased the mitochondrial membrane potential (Fig. 4C) compared to non-cryopreserved cells (1.00 ± 0.62 AFU, Fig. 4D). The 2.5 % Me₂SO-sucrose was with the higher $\Delta\Psi_m$ (4.92 ± 1.23 AFU) when compared to the other solutions ranging from 3.24 AFU to 3.91 AFU.

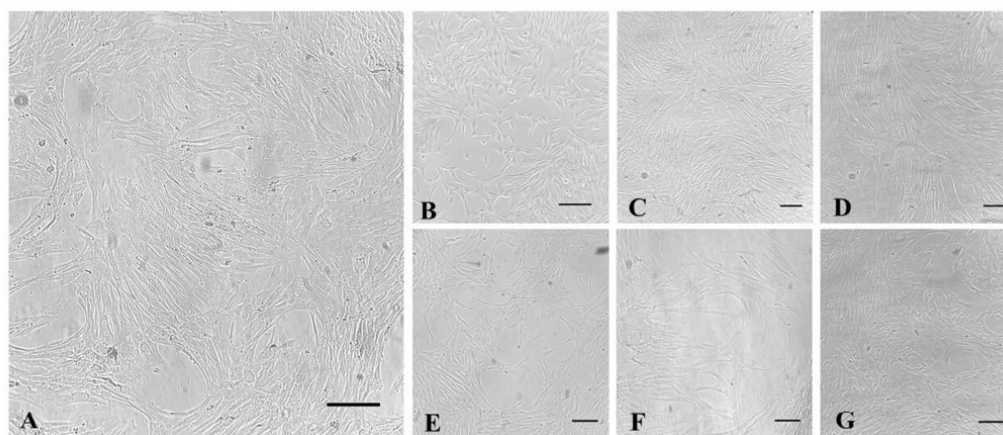


Fig. 1. Morphology evaluation of northern tiger cat fibroblasts subjected to cryopreservation in different solutions. (A) Non-cryopreserved cells and cells cryopreserved using (B) 2.5 % Me₂SO; (C) 10 % Me₂SO; (D) 2.5 % Me₂SO with sucrose; (E) 2.5 % Me₂SO with L-proline; (F) 10 % Me₂SO with sucrose; (G) 10 % Me₂SO with L-proline; Scale bars: 50 μm. Magnification: 20 × .

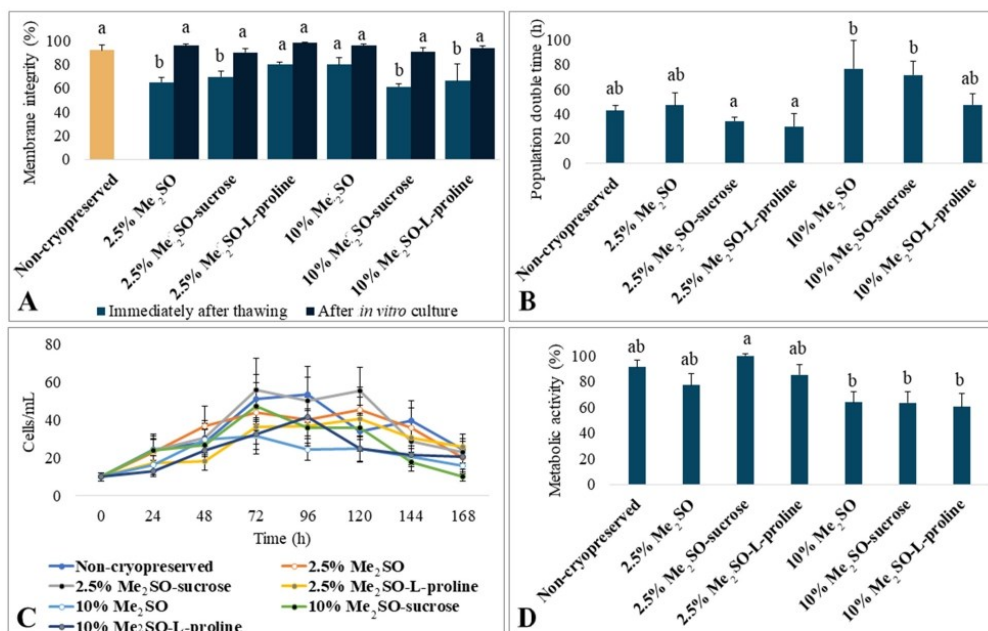


Fig. 2. Membrane integrity and proliferative and metabolic activities of northern tiger cat fibroblasts after cryopreservation using different cryoprotectant solutions. (A) Cell membrane integrity; (B) Population double time (PDT); (C) Growth curve after 7 days of cell culture; (D) Metabolic activity. ^{a,b} P < 0.05.

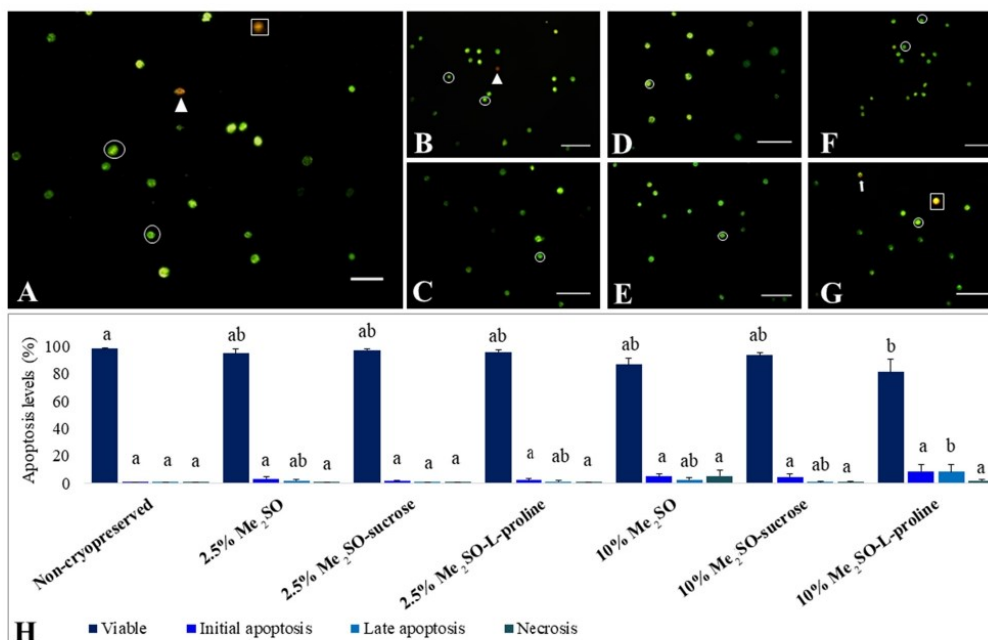


Fig. 3. Measurement of apoptotic levels of northern tiger cat fibroblasts after cryopreservation using different cryoprotectant solutions. Representative image of non-cryopreserved cells (A) and cells cryopreserved with 2.5 % Me₂SO (B); 10 % Me₂SO (C); 2.5 % Me₂SO with sucrose (D); 10 % Me₂SO with sucrose (E); 2.5 % Me₂SO with L-proline (F); 10 % Me₂SO with L-proline (G). Circle: Viable cell; Square: Initial apoptosis; Triangle: Late apoptosis; Arrow: Necrosis. (E) Quantification of apoptotic levels; Scale bars: 50 μ m. Magnification: 40 $\times$.

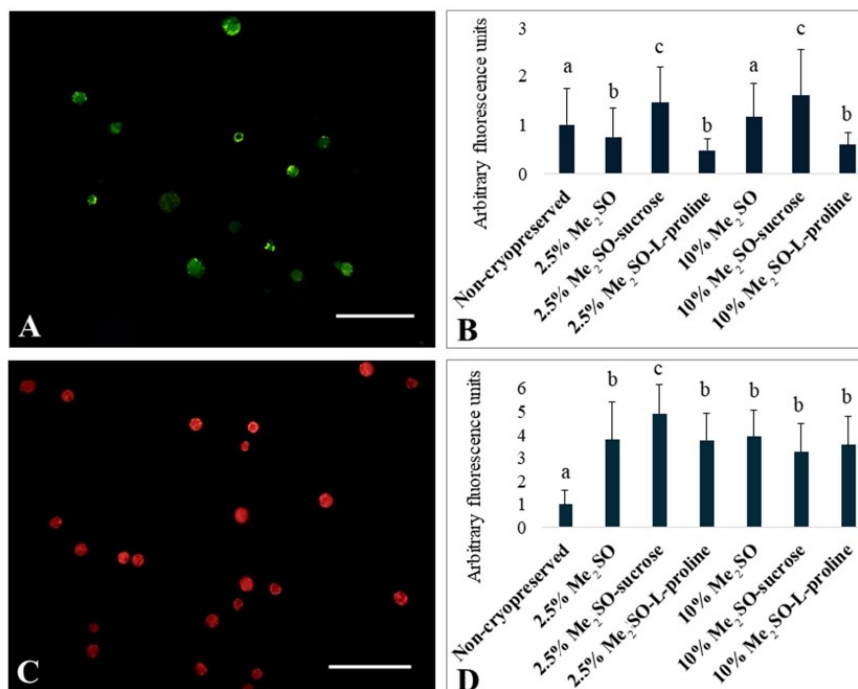


Fig. 4. Oxidative stress measured by the ROS levels and mitochondrial membrane potential ($\Delta\Psi_m$) of northern tiger cat fibroblasts after cryopreservation using different cryoprotectant solutions. (A) Non-cryopreserved cells marked with MitoTracker Red; (B) Mitochondrial membrane potential measurement; (C) Non-cryopreserved cells marked with H₂DCFDA; (D) Quantification of ROS levels. Scale bars: 50 μ m. Magnification: 40 $\times$. ^{a,b,c} $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In this study, we observed no synergistic effect of sucrose and L-proline with reducing the concentration of Me₂SO on the cryosurvival of northern tiger cat fibroblasts. In wild felids, adding extracellular cryoprotectants, such as sucrose, can improve cells' metabolic activity and PDT [17]; however, this effect was not observed in northern tiger cat somatic cells. Reducing cryoprotectant concentration has been recognized as a strategy to decrease the toxicity during cell cryopreservation. Arantes et al. [1] reported >80 % of viable somatic cells after cryopreservation with 2.5 % Me₂SO of northern tiger cats. In our study, the viability of cells cryopreserved with 2.5 % Me₂SO decreased immediately after thawing. Klbik et al. [13] described the effect of a lower concentration of Me₂SO (~2 %) causing a more significant ice crystal formation and decreasing cell viability by exposure to higher pressure. The influence of Me₂SO concentration is clear, due to the 10 % Me₂SO likely preserved higher viability immediately after thawing for northern tiger cat somatic cells.

Moreover, the presence of sucrose did not improve the viability immediately after thawing. Similar results were observed for adult skin fibroblasts of Iberian lynx [17]. Fortunately, the addition of 100 mM L-proline to 2.5 % Me₂SO improved this somatic cell viability. Bailey et al. [3] showed that 100 mM L-proline inhibited ice crystal development, increasing the viability from 25 % to 60 % in human lung carcinoma cells. This ability is especially dependent on extracellular cryoprotectant characteristic as hydration ability, cryoprotectant concentration, and the type and species [5].

Interestingly, all the groups promoted the conservation of metabolic activity. Whaley et al. [31] described the function of Me₂SO as decreasing the membrane thickness, resulting in a permeability increase

and promoting less intracellular ice formation. This mechanism could improve cell cryosurvival even without combining cryoprotectants. Moreover, the solution with 2.5 % or 10 % Me₂SO alone or with sucrose protected the metabolic activity of puma fibroblasts [28].

Additionally, great results were reported on cell proliferation of northern tiger cat cells in our study. Chaytor et al. [5] described the Me₂SO action as inhibiting ice recrystallization at lower concentrations (2.5 %), especially when combined saccharides. Moreover, the cryopreservation of Jurkat cells with 5 % Me₂SO and 100 mM L-proline maintained cell proliferation, similar to our study with both Me₂SO concentrations [21].

Furthermore, the antioxidant effect of L-proline in our study acted decreasing ROS levels and increased $\Delta\Psi_m$ in all solutions with Me₂SO. Similarly, Len et al. [15] described that L-proline during cryopreservation act reducing ROS levels by connecting with free radicals and increasing the $\Delta\Psi_m$. Inversely, sucrose increased the ROS levels. These levels can be caused by Me₂SO promoting the permeability of the cell membrane, an increase of sucrose can pass through the membrane, resulting in cell stress [9,23]. Unlike the solution with extracellular cryoprotectant or only 2.5 % Me₂SO, the solution with 10 % Me₂SO maintained the ROS levels. These results occur due to the Me₂SO intracellular protective action, probably decreasing the ice growth and re-crystallization during thawing [4,11].

Unfortunately, L-proline resulted in a decrease in viable cells by apoptosis levels assay. L-proline has been used as a substitute for Me₂SO to avoid the toxicity effect and promote conservation with a substance with similar osmolality [6,19]. In our study, the increase of Me₂SO concentration in the presence of L-proline could have changed the osmotic equilibrium during the dehydration of slow freezing and affected the protein stability, resulting in cell death [32].

Finally, adding L-proline or sucrose in solution with 2.5 % or 10 % Me₂SO did not improve the cell parameters compared to control groups with only Me₂SO. Arantes et al. [1] described the success of northern tiger cat somatic cell cryopreservation with a solution without Me₂SO or extracellular cryoprotectant; northern tiger cat somatic cells probably presented better results with simpler solutions. Awan et al. [2] showed that Me₂SO can have a variable effect on different cell types, concentrations, and mechanisms, resulting in better results alone than when combined with other cryoprotectants.

5. Conclusions

Even with several studies showing the efficiency of sucrose or L-proline on somatic cell cryopreservation, adding these substances did not improve the cryopreservation of northern tiger cat fibroblasts. Moreover, reducing the concentration of Me₂SO to 2.5 % had no impact on the efficiency of cryopreservation. Therefore, 10 % Me₂SO was determined to be most effective for cryopreserving northern tiger cat fibroblasts.

CRedit authorship contribution statement

João Vitor da Silva Viana: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Lhara Ricarlany Medeiros de Oliveira: Methodology, Investigation. Luanna Lorena Vieira Rodrigues: Methodology, Investigation. Yasmin Beatriz França Moura: Methodology. Antonia Beatriz Mendonça Pereira: Methodology. Patrícia Vasconcelos Alves: Methodology. Herlon Victor Rodrigues Silva: Methodology. Alexsandra Fernandes Pereira: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank the Zoos in Ceará for the animal care and the availability of animal access. This study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES, Financial Code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, no. 309078/2021-0). A.F. Pereira was a CNPq researcher.

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3053 ANEXO No. 5: COMPROVANTE DE ACEITE DO ARTIGO DE REVISÃO: THE ROLE OF
 3054 SOMATIC CELL SYNCHRONIZATION IN NUCLEAR TRANSFER AND INDUCED
 3055 PLURIPOTENT STEM CELLS FOR WILD FELIDS

28/02/2025, 19:38

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3057 ANEXO No. 6: COMPROVANTE DE SUBMISSÃO DO ARTIGO EXPERIMENTAL:
 3058 EXPLORING THE EFFECTS OF ROSCOVITINE, SERUM STARVATION, AND CONTACT
 3059 INHIBITION AT G₀/G₁ ARREST IN NORTHERN TIGER CAT DERMAL FIBROBLASTS.

3060

28/02/2025, 19:42 Gmail - IVAN: Your manuscript entitled Exploring the effects of roscovitine, serum starvation, and contact inhibition at G₀/G₁ a...



João <joaovitorvianajr@gmail.com>

IVAN: Your manuscript entitled Exploring the effects of roscovitine, serum starvation, and contact inhibition at G₀/G₁ arrest in northern tiger cat dermal fibroblasts - [EMID:c55fc142bf2fb9d2]

1 mensagem

Tetsuji Okamoto <em@editorialmanager.com>

17 de fevereiro de 2025 às 19:18

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Para: João Vitor da Silva Viana <joaovitorvianajr@gmail.com>

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Ref.:

Ms. No. IVAN-D-25-00030

Exploring the effects of roscovitine, serum starvation, and contact inhibition at G₀/G₁ arrest in northern tiger cat dermal fibroblasts

In Vitro Cellular & Developmental Biology - Animal

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28/02/2025, 19:42

Gmail - IVAN: Your manuscript entitled Exploring the effects of roscovitine, serum starvation, and contact inhibition at G0/G1 a...

Reviewers' comments:

Reviewer #1: IVAN-D-25-00030 Exploring the effects of roscovitine, serum starvation, and contact inhibition at G0/G1 arrest in northern tiger cat dermal fibroblasts. Corresponding author: Alexandra Fernandes Pereira, Ph.D.

This manuscript reports the efforts to increase the ration of G0/G1 in populations of cells derived from Northern Tiger Cat dermis. Three methods are reported: roscovitine treatment, serum starvation and contact inhibition. Of the three methods, serum starvation produced the highest arrest in the populations.

Review:

1) Introduction: Line 59: please consider changing "crucial points" to "techniques"

Materials and Methods:

2) Lines 108-114: The description of the establishment of the cell strains is very limited and insufficient for this journal. Please consider reviewing the similar section in the Veragas et al 2017 publication as an example of a more complete text for this section.

3) Lines 116 to 121: Please provide more detail on the cryopreservation procedures (freezing and thawing). Please provide relative viabilities and how they were determined.

4) Lines 149-150: It is assumed that the fibroblasts were cultured as attached cells. Please provide details as to how the cells were harvested from the plastic.

5) Lines 164-172: Again, these cells were trypsinized off of the plastic to form the cell suspension. We have very limited detail. The reader must assume that the monolayers were rinsed to remove the FBS. Therefore, only the adherent cells would have been evaluated for cell viability. This is very important as fibroblasts that are dead or dying do not adhere to the plastic substrate and would be lost for the cell counting (percent viability) determinations. To this end, it is critical to absolute cell counts on each treatment group to know how many cells were lost (dead). Was this done and if so, the reader needs to know the cell numbers compared to the control cultures. Please add this information.

6) Lines 174-180: The specificity of this method for apoptosis (compared to necrosis) is based on the Veraguas et al 2017 publication. However, that publication provided no substantiation to support the specificity of the methods. That specificity need to be provided from some other source. Please add that documentation.

Results:

7) Line 195: "the animals showed a standard growth curve" please correct.

8) Lines 199-204, 229-235, and 244-249: these data require that the points raised in 4 above be addressed.

9) The apoptosis data required that point 5 be addressed.

Discussion:

10) What would be an acceptable target percentage of cells in the G0/G1 arrest to allow the cells to be used for species conservation?

Figures:

11) The photomicrographs of the cells in monolayer culture are not really of publication quality (very hard to see real differences). Please consider how these might be improved (really contrast is needed to better see the cells). Of importance, whatever is done must be done to all the micrographs in the manuscript and the method needs to be detailed in the Materials and Methods.

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3084 ANEXO A-CERTIFICADO DE REVISÃO PARA LÍNGUA INGLESA DO ARTIGO 01:
 3085 ESTABLISHMENT AND CHARACTERIZATION OF FIBROBLASTS CELL LINES FROM
 3086 NOTHERN TIGER CAT (*Leopardus tigrinus*, Schreber, 1775) DURING EXTENDED
 3087 PASSAGE AND CRYOPRESERVATION. EMPRESA: CIA DAS TRADUÇÕES - 31/03/2024.



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3090 **ANEXO B-CERTIFICADO DE REVISÃO PARA LÍNGUA INGLESA DO ARTIGO 02: NO**
 3091 **SYNERGISTIC EFFECT OF NATURAL CRYOPROTECTANTS WITH DIMETHYL**
 3092 **SULFOXIDE IN THE CONSERVATION OF NOTHERN TIGER CAT FIBROBLASTS.**
 3093 **EMPRESA: CIA DAS TRADUÇÕES - 24/07/2024.**

REVIEW

CERTIFICATE

To whom it may concern:

We hereby declare that the manuscript described below

Title of the Paper: No synergistic effect of natural cryoprotectants with dimethyl sulfoxide in the conservation of northern tiger cat fibroblasts

Authors: João Vitor da Silva Viana, Lhara Ricarlany Medeiros de Oliveira, Luanna Lorena Vieira Rodrigues, Yasmin Beatriz França Moura, Antonia Beatriz Mendonça Pereira, Patrícia Vasconcelos Alves, Herlon Victor Rodrigues Silva, Alexsandra Fernandes Pereira

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We testify that the manuscript was carefully reviewed for being clearer and with the proper scientific language in English, including grammar, punctuation, spelling and general style. However, the study content and the authors' purposes, as understood from the original text, were not changed anyhow.

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3095 **ANEXO C-CERTIFICADO DE REVISÃO PARA LÍNGUA INGLESA DO ARTIGO 03: THE**
 3096 **ROLE OF SOMATIC CELL SYNCHRONIZATION IN NUCLEAR TRANSFER AND**
 3097 **INDUCED PLURIPOTENT STEM CELLS FOR WILD FELIDS. EMPRESA: EDITAGE -**
 3098 **06/02/2025.**



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MANUSCRIPT TITLE

The role of somatic cell synchronization in nuclear transfer and induced pluripotent stem cells for wild felids

AUTHORS

Alexsandra Fernandes Pereira, João Vitor da Silva Viana

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- 3100 **ANEXO D-CERTIFICADO DE REVISÃO PARA LÍNGUA INGLESA DO ARTIGO 04:**
- 3101 **EXPLORING THE EFFECTS OF ROSCOVITINE, SERUM STARVATION, AND CONTACT**
- 3102 **INHIBITION AT G₀/G₁ ARREST IN NORTHERN TIGER CAT DERMAL FIBROBLASTS.**
- 3103 **EMPRESA: CIA DAS TRADUÇÕES.**

REVIEW

CERTIFICATE

To whom it may concern:

We hereby declare that the manuscript described below

Title of the Paper: EXPLORING THE EFFECTS OF ROSCOVITINE, SERUM STARVATION, AND CONTACT INHIBITION AT G₀/G₁ ARREST IN NORTHERN TIGER CAT DERMAL FIBROBLASTS

Authors: João Vitor da Silva Viana, Brenna de Sousa Barbosa, Luanna Lorena Vieira Rodrigues, Antonia Beatriz Mendonça Pereira, Patrícia Vasconcelos Alves, Herlon Victor Rodrigues Silva, Sarah Sant'Anna Maranhão, Carlos Roberto Koscky Paier, Maria Claudia dos Santos Luciano, Cláudia Pessoa4, Alexandra Fernandes Pereira

Was reviewed into the English language and edited for being clearer, according to the scientific writing, by the professional scientific translation team from Cia das Traduções (MS Traduções Ltda.- National Registry of Legal Entities – CNPJ n. 08.966.620/0001-91).

We testify that the manuscript was carefully reviewed for being clearer and with the proper scientific language in English, including grammar, punctuation, spelling and general style. However, the study content and the authors' purposes, as understood from the original text, were not changed anyhow.

The respective author read the manuscript reviewed into English by us before the final review, and the same had the opportunity of selecting the terms and expressions suggested by the reviewer in case of doubts, so that the final version was adjusted according to his intentions and prepared for publishing.

A copy of both reviews of the original manuscript sent by the client and the review made by Cia das Traduções will be made available under request, so that it is verified, within minimum time period of one year.



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Manager
Cia das Traduções

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3105 **ANEXO E - COMPROVANTE DA AUTORIZAÇÃO DE ATIVIDADES COM FINALIDADE**
 3106 **CIENTÍFICA EMITIDA PELO ICMBio NO SISTEMA SISBIO, REQUISITO PARA A**
 3107 **COLHEITA DAS AMOSTRAS DOS ANIMAIS.**



Ministério do Meio Ambiente - MMA
 Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
 Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 88201-1	Data da Emissão: 03/07/2023 23:50:40	Data da Revalidação*: 03/07/2024
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Alexandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: Avaliação das condições de cultivo in vitro, criopreservação e sincronização em G0/G1 de células somáticas derivadas do gato-do-mato-pequeno (<i>Leopardus tigrinus</i> Schreber, 1775)	
Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta das amostras de pele auricular	05/2023	01/2024

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Herlon Victor Rodrigues Silva	Colaborador	027.343.873-52	Brasileira

Observações e ressalvas

1	Todos os membros da equipe de pesquisa devem estar cientes das recomendações e boas práticas a serem seguidas neste momento de emergência zoonossanitária no Brasil devido à gripe aviária. Informe-se na página do CEMAVE na Internet: https://www.gov.br/icmbio/pt-br/assuntos/centros-de-pesquisa/cehave/destaques/gripe-aviaria/gripe-aviaria-1 .
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Página 1/4

3109 **ANEXO F - COMPROVANTE DO PARECER TÉCNICO DO CEUA/UFERSA**
 3110 **APROVANDO A REALIZAÇÃO DO PROJETO DE PESQUISA, REQUISITO PARA A**
 3111 **COLHEITA DAS AMOSTRAS DOS ANIMAIS.**



UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Mossoró, 2 de maio de 2023.

PARECER 20/2023

*Certificamos que o projeto intitulado "Avaliação das condições de cultivo in vitro, criopreservação e sincronização em G0/G1 de células somáticas recuperadas do gato-do-mato-pequeno (*Leopardus tigrinus* Schreber, 1775)" sob a responsabilidade de Alexsandra Fernandes Pereira, o qual envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da lei 11.794 de 8 de outubro de 2009 e com as normas editadas pelo Conselho Nacional de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UFERSA em reunião no dia 14 de abril de 2023.*

Vigência do projeto	Maio a agosto/2023		
Espécie/linhagem	Leopardus tigrinus		
N. de Animais	2	1	2
Peso/idade	Sem restrição por peso / adultos		
Sexo	Macho e fêmea	macho	macho
Origem	Zoológico São Francisco das Chagas / Canindé-CE	ECOPOINT parque ecológico / Fortaleza-CE	Zoológico Sargento Pprata / Fortaleza-CE

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3115 ANEXO G -RESUMO SUBMETIDO AO SIMPÓSIO DE PÓS-GRADUAÇÃO EM CIÊNCIA
3116 ANIMAL, REALIZADO PRESENCIALMENTE DE 7 A 8 DE DEZEMBRO DE 2023

3117 **ISOLAMENTO E CULTIVO *IN VITRO* DE CÉLULAS SOMÁTICAS DERIVADAS DA**
3118 **PELE AURICULAR DE GATO-DO-MATO-PEQUENO**
3119 *ISOLATION AND IN VITRO CULTURE OF SOMATIC CELLS DERIVED FROM THE*
3120 *EAR SKIN OF ONCILLA*

3121
3122 *Morfofisiologia e Biotecnologia Animal*

3123
3124 J.V.S. VIANA^{1*}, A.F. PEREIRA¹

3125 1 – Laboratório de Biotecnologia Animal (LBA), Universidade Federal Rural do Semi-Árido,
3126 UFERSA, Mossoró, RN.
3127 joaovitorvianajr@gmail.com
3128

3129 **Objetivos:** Descrever o isolamento e cultivo *in vitro* de células somáticas de gato-do-mato-
3130 pequeno (*Leopardus tigrinus* Schreber, 1775). **Material e Métodos:** Biópsias de pele da região
3131 auricular periférica foram recuperadas com auxílio de tesoura cirúrgica de quatro indivíduos
3132 adultos, anestesiados e provenientes de zoológicos localizados no Estado do Ceará. As amostras
3133 foram transportadas por 3 a 6 h em meio essencial mínimo modificado por Dulbecco (DMEM)
3134 suplementado com 10% de soro fetal bovino e 2% de solução de antibióticos-antimicóticos, a
3135 4°C. No laboratório, fragmentos (9,0 mm³) foram cultivados em atmosfera controlada (38,5°C,
3136 6,5% de CO₂) e avaliados a cada 24 h usando microscópio invertido. Após as células atingirem
3137 70% de confluência, foi realizado o primeiro subcultivo celular para a avaliação da viabilidade
3138 pelo ensaio de azul de tripan e atividade metabólica pelo ensaio do brometo de 3-(4,5-dimetil-2-
3139 tiazolil)-2,5-difenil-2H-tetrazólio. Todos os dados foram apresentados descritivamente como
3140 média ± erro padrão. **Resultados:** O tempo de cultivo total foi de 16 ± 0,5 dias para as amostras
3141 derivadas de todos os indivíduos e dos dezesseis fragmentos submetidos ao cultivo (quatro
3142 fragmentos por animal) todos apresentaram aderência em até 2,0 ± 1,5 dias, com 88% dos
3143 fragmentos apresentando subconfluência celular a partir de 8,5 ± 0,5 dias. Em geral, a partir da
3144 análise morfológica, todas as células apresentaram morfologia fusiforme com núcleo oval
3145 centralizado, mostrando-se semelhante a fibroblastos. Quanto ao ensaio de azul de tripan, foi
3146 obtida uma viabilidade de 94,5% ± 0,01. Já no ensaio de atividade metabólica foi obtido o
3147 percentual de 95,8% ± 20,2. **Conclusões:** Biópsias da região auricular periférica de gato-do-
3148 mato-pequeno permitiram o isolamento de células viáveis semelhantes a fibroblastos e com alta
3149 funcionalidade metabólica, visando à conservação genética desta espécie.

3150
3151 **Palavras-chave:** felídeos silvestres, cultivo celular, criobanco.

3152
3153 **Keywords:** wild felids, cell culture, cryobank.

3154
3155 **Protocolo CEUA:** no. 20/2023. **Protocolo ICMBio:** no. 88201-1.

3156
3157 **Fonte de Fomento:** CAPES, bolsa de mestrado, processo no. 88887.828217/2023-00; CNPq,
3158 auxílio à pesquisa, processo no. 309078/2021-0.

- 3159 **ANEXO H -RESUMO SUBMETIDO AO X INTERNATIONAL SYMPOSIUM ON ANIMAL**
 3160 **BIOLOGY OF REPRODUCTION, REALIZADO PRESENCIALMENTE DE 1 e 2 DE JULHO**
 3161 **DE 2024.**



X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Isolation and extended *in vitro* culture of fibroblasts from northern tiger cat (*Leopardus tigrinus* Schreber, 1775) as a step towards for somatic cell nuclear transfer

João Vitor da Silva Viana¹, Lhara Ricarlany Medeiros de Oliveira¹, Luanna Lorena Vieira Rodrigues¹, Yara Letícia Frutuoso e Silva¹, Ana Livia Rocha Rodrigues¹, Patrícia Vasconcelos Alves², Herlon Victor Rodrigues Silva³, Alessandra Fernandes Pereira¹

¹Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoró, Brazil

²Zoológico Municipal Sargento Prata, Fortaleza, Brazil

³Laboratory of Carnivores Reproduction, University of Ceará State (UECE), Fortaleza, Brazil

E-mail: joaovitorvianajr@gmail.com

Financial support: CNPq and CAPES

The establishment of *in vitro* culture of cells derived from skin fragments has been proposed as a useful technique for the conservation of endangered species. Among the species vulnerable to extinction, the northern tiger cat requires strategies for the conservation of its genetic diversity. An interesting conservation strategy would be one aimed at the development of cryobanks for use in assisted reproduction techniques. In general, the characterization of the cells during extended culture represents an important step in the formation of these banks. Therefore, we described the extended *in vitro* culture of fibroblasts derived from northern tiger cat skin. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 20/2023) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 88201-1). Then, skin biopsy (1.0 cm²) derived from ear region was recovered from one male of Sargento Prata Municipal Zoo (Fortaleza, Brazil), after anesthetized. In the laboratory, fragments (6.0 mm³) were cultured under controlled atmosphere (38.5°C, 6.5% CO₂) in minimal essential medium modified by Dulbecco (DMEM) supplemented with 10% fetal bovine serum and 2% antibiotic-antimycotic solution and monitored every 24 h. Cells isolated from tissues were evaluated after the third, seventh and eleventh passage for viability with trypan blue assay, metabolic activity using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide, proliferative activity using population double time (PDT), apoptotic levels with acridine orange and ethidium bromide, reactive oxygen species (ROS) levels and mitochondrial membrane potential ($\Delta\Psi$ m) using H2DCFDA and MitoTracker Red probes, respectively. Additionally, cells in the third passage were evaluated for identification of your karyotype and confirmation of cell type using primary anti-vimentin antibody. All data were presented descriptively. Thus, the total culture time was 20 days for the samples derived from a male. The initial four fragments submitted to the culture all presented adherence on the first day with cell growth around the explants from the eighth day. Cells reached 70% confluence forming monolayer on day 17. During the third passage, the cells were analyzed with primary anti-vimentin antibody, identifying them as fibroblasts based on the presence of intermediate filaments. Moreover, karyotype evaluation was in conformance with previous reports on this species, with 2n = 36 chromosomes, 34 of which were autosomal, plus 2 sex chromosomes. Regarding to viability using trypan blue, values above 80% were observed in all passages (third: 80%, seventh: 97%, and eleventh: 82%). However, a numerical reduction was observed in metabolic activity during passages (third: 79.3%, seventh: 79.3%, and eleventh: 27.4%). Moreover, PDT values were similar between the third (31.7 h) and seventh passages (29.0 h), while due to the low cell concentration presented in the eleventh passage, this parameter was not quantified. Regarding to apoptosis levels, values above 95% were observed in all passages for viable cells (third: 99%, seventh: 99%, and eleventh: 98%). Additionally, ROS levels, evaluated in arbitrary fluorescence units (AFU), were maintained in 1.00 AFU during all the passages. Values of $\Delta\Psi$ m varied between passages (third: 1.23 AFU, seventh: 2.81 AFU, and eleventh: 2.68 AFU). In summary, we were able to isolate somatic cells derived from northern tiger cat skin, associated with high viability rates after extended *in vitro* culture. These results described, at first, the cellular characteristics according to different quality parameters of Northern tiger cat samples during extended *in vitro* culture, providing a valuable source for assisted reproduction techniques, aiming at the genetic conservation of this species.

ANEXO I -RESUMO SUBMETIDO AO XXVI CONGRESSO BRASILEIRO DE
REPRODUÇÃO ANIMAL, SERÁ REALIZADO PRESENCIALMENTE DE 7 a 9 DE MAIO
DE 2025.

The impact of roscovitine exposure time on G₀/G₁ cell cycle arrest and apoptotic levels in fibroblasts of the northern tiger cat

O impacto do tempo de exposição a roscovitina na parada do ciclo celular G₀/G₁ e nos níveis apoptóticos em fibroblastos de gato-do-mato-pequeno

**João Vitor da Silva Viana^{1*}, Luanna Lorena Vieira Rodrigues¹, Brenna de Sousa Barbosa¹,
Patrícia Vasconcelos Alves², Herlon Victor Rodrigues Silva³, Sarah Sant'Anna Maranhão⁴, Claudia
Pessoa⁴, Alessandra Fernandes Pereira¹**

¹Laboratório de Biotecnologia Animal, Universidade Federal Rural do Semi-Árido, Mossoró/RN, Brasil; ²Zoológico Municipal Sargento Prata, Fortaleza/CE, Brasil. ³Laboratório de Reprodução de Carnívoros, Universidade Federal do Ceará, Fortaleza/CE, Brasil.; ⁴Laboratório de Oncologia Experimental, Universidade Federal do Ceará, Fortaleza/CE, Brasil.

*E-mail: joaovitorvianajr@gmail.com

The northern tiger cat plays a fundamental role in maintaining ecosystem balance; however, it is vulnerable to extinction due to human interference. In this context, the development of reproductive biotechnologies, such as somatic cell nuclear transfer (SCNT), is crucial. To enhance SCNT efficiency, it is essential to establish G₀/G₁ cell cycle arrest. Among the available methods, roscovitine (RSV) is notable for its efficiency in inducing cell cycle arrest while minimizing viability loss and DNA fragmentation. However, the efficacy of RSV is influenced by exposure time, which varies depending on species-specific cellular characteristics. Thus, this study aimed to evaluate the impact of roscovitine exposure time on G₀/G₁ cell cycle arrest and apoptotic levels in fibroblasts of the northern tiger cat. Initially, skin biopsies were collected from the ear region of four northern tiger cats and cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and 2% penicillin-streptomycin solution at 38.5 °C and 6.5% CO₂. At the fourth passage, once fibroblasts reached 50–60% confluence, they were divided into a cycling control group and treatment groups exposed to 7.5 µM RSV for 24 or 48 hours. Apoptotic levels were assessed using acridine orange and ethidium bromide staining, while cell cycle phases were determined by flow cytometry. For each sample, 15,000 events were recorded, and histograms were generated to analyze the percentage of cells in each cell cycle phase (G₀/G₁, S, G₂/M) using MODFIT software (version 5.0). Data were expressed as mean ± standard error and analyzed using ANOVA followed by Tukey's test (P < 0.05). An increase in RSV exposure time from 24 h (94.3% ± 2.8) to 48 h (89.0% ± 3.7) resulted in a decrease in viable cells compared to cycling cells (99.8% ± 0.3). Both exposure times, 24 h (4.5% ± 2.1) and 48 h (4.5% ± 0.8), led to an increase in early apoptotic cells compared to cycling cells (0.3% ± 0.3). Moreover, 48 h of RSV treatment significantly increased the percentage of late apoptotic cells (3.5% ± 0.6) compared to 24 h (1.0% ± 0.5) and cycling cells (0.0% ± 0.0). Regarding cell cycle distribution, the increase in RSV exposure time from 24 h (83.6% ± 1.2) to 48 h (84.4% ± 1.7) did not significantly affect the G₀/G₁ cell population, which remained similar to that of cycling cells (86.0% ± 0.9). Interestingly, 48 h of RSV treatment increased the S-phase cell population from 6.3% ± 0.5 to 8.5% ± 0.7, whereas the 24 h treatment maintained it around 6%. The G₂/M population remained unchanged across all groups at approximately 7%. In conclusion, RSV exposure time influences apoptotic levels without significantly enhancing G₀/G₁ cell cycle arrest. These findings

3209 highlight the need to explore alternative approaches, such as different chemical agents or varying RSV
3210 concentrations, to improve G₀/G₁ arrest in northern tiger cat fibroblasts and optimize SCNT protocols.

3211

3212 **Keywords:** wildlife conservation; chemical inhibition; cell cycle arrest.

3213 **Palavras-chave:** *conservação da vida selvagem; inibidor químico; parada do ciclo celular.*

3214